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New Strategies to Investigate the Transport of Pathogenic Microorganisms in the Subsurface

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Abstract

Of the world's liquid freshwater resources, 97% is stored as groundwater. For millennia, humans have used this resource to provide water for domestic use, agriculture and industry. The water quality of groundwater for drinking water, specifically, is important to human health and therefore the health of society. The focus of this doctoral thesis is on the microbial water quality of groundwater intended for drinking water consumption.

The presence and transport of pathogenic microorganisms in the subsurface is difficult to detect and quantify due to their low concentrations in the environment. It is possible to perform field tests by injecting high concentrations of a colloidal tracer and taking measurements along the groundwater flow path; however, to ensure groundwater protection, it is not allowed to inject real pathogenic microorganisms. Surrogates that are representative of the microorganisms under consideration need to be developed. The aim of this doctoral thesis is twofold: to study microbial transport in the subsurface by developing surrogates for two common and persistent pathogenic microorganisms, *Cryptosporidium parvum* and human adenovirus, and to use an appropriate method for detection and quantification of low concentrations of microorganisms and surrogates in environmental water.

Following the Introduction, Chapter 2 addresses the problem of detecting low concentrations of colloids in groundwater, as compared to surface water and a control of sterile deionized water. It was found that surrogates could be detected in very turbid lake water down to a size of 0.75 µm and a maximum sample volume of 1 ml. In pristine spring water, colloids could be quantified down to a size of 0.5 µm in a maximum volume of 500 ml. Chapter 3 goes on to use the method developed in Chapter 2 to enumerate low concentrations of surrogates representing *C. parvum* in laboratory column tests with granular limestone aquifer material. The best surrogate for *C. parvum*, as indicated by parameters calculated using colloid filtration theory and breakthrough curves, was a glycoprotein-coated microsphere. Surrogates representing human adenovirus were compared in Chapter 4 in order to determine the most ideal surrogate of those tested and the removal mechanism in limestone material. PRD1 phage was found to be the surrogate best representing human adenovirus removal in high carbonate material, but failed to mimic the detachment of human adenovirus under high ionic strength and high pH conditions.

In this doctoral thesis, two strategies were developed to further advance colloid tracer technology and the enumeration of colloids. The implication of this work is that each system comprising of aquifer media, certain chemical conditions and a specific microorganism is unique and needs to be considered on a case by case basis. Due to the complexity of the system, microbial transport in one material cannot be generalized to represent transport in all groundwater systems. This makes it almost impossible to establish standard methods to monitor drinking water quality in the subsurface. In conclusion, experimental field tests using a surrogate that is compared to the pathogenic microorganism in laboratory tests is recommended. With the results obtained from such tests, the transport processes of pathogenic microorganisms in groundwater can be better characterized and this would enable a risk approach analysis to be used.

Kurzfassung

Von den weltweit vorhandenen Süßwasservorkommen sind 97% als Grundwasser gespeichert. Über Jahrtausende haben Menschen Grundwasser im Haushalt, in der Landwirtschaft und in der Industrie verwendet. Die Wasserqualität des Grundwassers ist, wenn es als Trinkwasser genutzt wird, besonders wichtig und beeinflusst in hohem Maße die menschliche Gesundheit und ist damit von großer gesellschaftlicher Bedeutung. Der Schwerpunkt dieser Doktorarbeit liegt in der Erfassung der mikrobiellen Wasserqualität des Grundwassers für den Gebrauch als Trinkwasser.

Aufgrund ihrer geringen Konzentrationen in der Umwelt ist es schwierig, das Vorhandensein und den Transport von pathogenen Mikroorganismen im Untergrund zu messen und zu quantifizieren. Es ist möglich, Feldversuche durch Injektion kolloidaler Tracer in hohen Konzentrationen und Messungen entlang des Grundwasserfließweges durchzuführen. Um einen Grundwasserschutz zu gewährleisten, ist es jedoch nicht erlaubt pathogene Mikroorganismen in der Umwelt einzusetzen. Ersatzstoffe (Surrogate), die ein ähnliches Transportverhalten wie Mikroorganismen haben, sind daher in Entwicklung bzw. wurden bereits entwickelt. Das Ziel dieser Dissertation ist es, die Eignung von bereits entwickelten Surrogate für zwei häufige vorkommende pathogene Mikroorganismen (*Cryptosporidium parvum* und humaner Adenovirus) auf ihre Verwendung zur Beschreibung des Transportes dieser Mikroorganismen im Untergrund zu untersuchen. Ein weiteres Ziel ist es, eine geeignete Methode zur Detektion und Quantifizierung von Mikroorganismen zu finden, die in niedrigen Konzentrationen in der Umwelt vorkommen, was häufig bei pathogenen Mikroorganismen der Fall ist.

Nach der Einführung in die Thematik wird im Kapitel 2 das Problem der Detektion geringer Konzentrationen von Kolloiden im Grundwasser im Vergleich zu Oberflächenwasser und einer Probe mit sterilem deionisiertem Wasser behandelt. Es wurde gezeigt, dass die verwendeten Surrogate in sehr trübem Wasser eines Sees bis zu einer Größe von 0,75 µm und einem maximalen Probenvolumen von 1 ml nachgewiesen werden können. Bei reinem Quellwasser kann bis zu einem maximalen Probenvolumen von 500 ml Kolloide bis zu einer Größe von 0,5 µm quantifiziert werden. Das Kapitel 3 behandelt die Untersuchung verschiedener Surrogate für *Cryptosporidium parvum* in Säulenversuchen im Labor. Dabei wurde die Surrogate untersucht, ob diese bei niedrigen Konzentrationen im körnigen

Kalkstein verwendet werden können und welche am besten den Transport im Untergrund beschreiben. Dabei zeigte sich, dass der beste Ersatz für *Cryptosporidium parvum* auf Basis der Berechnungen mit der Kolloid Filtrationstheorie Glykoprotein-beschichtete Mikrosphären sind. Surrogate, welche humane Adenoviren repräsentieren wurden in Kapitel 4 untersucht, um das beste Surrogat für das Reduktionsverhalten in Kalkstein zu ermitteln. Es wurde festgestellt, dass die Phage PRD1 bei hohem Kalkgehalt am besten humane Adenoviren repräsentieren. Jedoch konnte mit PRD1 die Ablösung des humanen Adenovirus bei hoher Ionenstärke und hohem pH-Gehalt nicht nachbildet werden.

Die Resultate dieser Arbeit zeigen, dass die für jeden Grundwasserkörper unterschiedlichen physikalischen und chemischen Bedingungen und das Verhalten der Mikroorganismen stark beeinflusst und von Fall zu Fall sehr unterschieldich sein kann. Aufgrund dieser Komplexität des Systems kann der mikrobielle Transport nicht nur von den vorhandenen Bodenmaterial abgeleitet werden, sondern hängt auch von dessen Eigenschaften und den vorkommenden Mikroorganismen ab. Eine Übertragung auf andere Grundwasserkörper ist daher sehr schwierig und erfordert in den meisten Fällen Untersuchungen vor Ort, wodurch die Ableitung der standardisierten Methode zur Überwachung der Trinkwasserqualität im Untergrund kaum möglich macht. Als Schlussfolgerung dieser Arbeit kann abgeleitet werden, dass geeignete Surrogate in Laborversuchen und in weiterer Folge auch bei Feldversuchen als Ersatz für pathogene Mikroorganismen angewendet werden können. Mit den bei diesen Versuchen gewonnen Ergebnisse können die Transportprozesse von pathogenen Mikroorganismen im Grundwasser wesentlich besser beschreiben werden und ermöglichen in weiterer Folge auch eine mikrobielle Risikoabschätzung.

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1 Introduction

Health is often equated with happiness and quality of life. A society with healthy individuals is more likely to be productive and stable, allowing citizens to participate in socially meaningful activities (Selin and Davey, 2012). Illnesses such as gastroenteritis and dysentery can result in such minor occurrences as a missed day at work to something as serious as death, if left untreated. Contaminated drinking water is a common cause of gastrointestinal disease, particularly in developing countries, where the danger of faecal contamination of drinking water sources is higher due to inadequate sewage disposal. Sickness resulting from contaminated drinking water is not, however, a problem that is limited to developing countries. In 1993, a cryptosporidiosis outbreak in Milwaukee, USA was estimated to have cost society US$96.2 million (WHO, 2004).

Drinking water sources include groundwater from pumping wells or natural springs and surface water sources ranging from large reservoirs to small-scale rain collection. Groundwater accounts for nearly one-third of drinking water used in Asia, which is inhabited by 60% of the world's population. Several of the world's most populous cities, such as Mexico City and Jakarta, depend on groundwater as the primary drinking water resource. The advantages of using groundwater as a drinking water resource, as opposed to surface water, are that water loss to evaporation is avoided and faecal contamination is minimized. One disadvantage is that groundwater may be exposed to certain geological formations that leach into the groundwater causing compounds that could cause diseases such as cancer. This was the case in Bangladesh where the high level of arsenic found in groundwater wells was identified in the 1990's as a hazard to human health. Shallow unconfined aquifers are also susceptible to microbial contamination or pollution from chemical compounds such as those used in fertilizers or pesticides for agricultural purposes. Therefore, it is imperative to define a protection zone around drinking water wells in order to protect this valuable resource.

The delineation of protection zones around groundwater wells intended for drinking water consumption is a challenging task for scientists and policy-makers. Many countries require a 50- or 60-day travel time (distance a point of contamination can travel in the groundwater over the course of 50 or 60 days) around a well in order to protect the drinking water

resource (e.g. Austria, Denmark, Germany). Others require more stringent protection, such as Oman or Australia, which define a 10-year travel time as a safe protection zone (WHO, 2006). The Netherlands has introduced an alternative approach basing the protection zone delineation on a risk assessment. Since it is not realistic that the risk of drinking water be zero, they have defined that an acceptable risk is one person per 10^4 persons per year becoming infected from drinking water consumption (WHO, 2006). This is now part of Dutch legislation, although protection of groundwater wells is still defined in the Netherlands by the 60-day travel time as being acceptable for pathogenic inactivation. Schijven *et al.* (2006) found that 6 to 12 times this travel time is more realistic for the required protection zones (1 to 2 years travel time) in a Dutch aquifer, but this depends on the specific adsorption processes of the site.

Ideally, each site should be considered individually. In some cases, the travel time may even be decreased if the attachment and removal mechanisms of the site merit it. The hypothesis of this work is that each aquifer is unique and each microorganism's reaction to the system is unique. In order to mimic a pathogenic microorganism, which cannot be used in the field, a surrogate needs to be developed. A surrogate is usually a colloid or another microorganism of similar size that is not dangerous to humans or the environment, but able to imitate the behaviour of the pathogen to some degree. Aspects that need to be considered when searching for an appropriate surrogate are: inactivation, adsorption to soil material, chemical reactions (geological chemistry), size, electrical charge, and hydrophobicity. The ideal surrogate for a pathogenic microorganism needs to be developed so that it is compatible with the porous media in question and represents transport in that system. More specifically, the goal of this doctoral thesis was to determine the transport of pathogenic microorganisms and their surrogates in material with a high carbonate content, such as limestone or gravel material, and to determine the removal mechanisms such as straining (due to size) or electrostatic interaction (due to surface charge).

Common surrogates for pathogenic microorganisms are fluorescent microspheres or nanoparticles and bacteriophages; the latter are viruses that are dangerous to bacteria but not infectious to humans. Fluorescent carboxylated polystyrene microspheres are often used to represent pathogenic microorganisms for practical reasons because they are relatively easy to enumerate and do not multiply or inactivate (Knappett et al., 2008), which can be an advantage depending on the research question. When using microspheres as a surrogate,

the inactivation rate of the target pathogen must be determined in separate experiments. Several different bacteriophages are used as model viruses due to their size as well as their surface characteristics and inactivation rates. Specifically, bacteriophages MS2 (27 nm in diameter, isoelectric point (pI) 3.5), PRD1 (62 nm, pI 3 - 4) and ϕX174 (27 nm, pI 6.6 - 6.8) have been used extensively in experiments in the field and column tests in the laboratory (Schijven and Hassanizadeh, 2000). MS2 phage is often used because it is considered a conservative surrogate and usually exhibits a low attachment rate under various conditions, which may be due to its low isoelectric point.

The advantage of using microspheres as a surrogate is that they are commercially available in many different sizes and can be coated with various substances in order to attain an isoelectric point that is similar to the pathogen in question. Microspheres with a carboxylated surface and the bacteriophages mentioned above that are traditionally used, are sometimes not the best surrogate to model microbial transport. For example, Harvey et al. (2011) reviewed various studies using carboxylated microspheres that were compared to *Cryptosporidium parvum* in column and field experiments and the authors concluded that they are not the ideal surrogate in many circumstances and need to be improved. Likewise, Redman et al. (1997) found that MS phage was not successful at mimicking the behaviour of Norwalk virus in column experiments, even though they are similar in size and shape. These studies, among others, indicate that there is a need for new and improved surrogates to advance research in the area of microbial transport in the subsurface.

One downfall of microspheres is that they are usually enumerated using methods such as microscopy or spectrofluorimetry which have high detection limits relative to the realistic concentration of pathogenic microorganisms found in groundwater. Due to the small size of bacteriophages, they are difficult to enumerate with microscopy, and are often enumerated using the culture method or quantitative chain reaction (qPCR). In theory, the culture method can detect one cell per petri dish and qPCR can detect one strand of DNA, although the detection limit is dependent on the method and the initial amount sampled, as well as the defined Poisson distribution for random sampling error. Therefore, the detection limit of bacteriophages is relatively low, and consequently, they cannot be directly compared to microspheres.

Chapter 2 attempts to address this discrepancy by testing an innovative method for detecting and enumerating microspheres using solid-phase cytometry. The low detection limit of this method allows a better comparison of microspheres to microorganisms. It was intended to push the limits of solid-phase cytometry in order to determine the smallest size of particle that could be detected in various background matrices. The matrices tested were samples taken from surface and groundwater sources in the natural environment. To find the limit of detection and quantification it was necessary to determine the maximum filtration volume that was possible to test before organic debris and particles caused the system to abort.

Chapter 3 builds on the method to enumerate microspheres using solid-phase cytometry by applying the technique to laboratory column tests using the pathogenic microorganism *Cryptosporidium parvum*, which causes cryptosporidiosis, and 4.5 µm microspheres as surrogates for comparison. Three different coatings for the microspheres were compared: the standard carboxyl surface, biotin protein and glycoprotein coatings. A previous study found that carboxylated microspheres were not successful at mimicking the transport of *C. parvum* in laboratory column tests using karst limestone aquifer material (Harvey et al., 2008). It was therefore attempted to test the usefulness of newly developed protein-coated microspheres by Pang et al. (2012) in granular limestone aquifer material.

Chapter 4 addresses the question of what the dominant removal mechanisms are in granular limestone porous media. This was done by comparing human adenovirus (HAdV) to three different surrogates in small-scale column tests: nanoparticles, MS2 and PRD1 bacteriophages. These three surrogates were chosen based on their size, morphology and surface charge characteristics. By comparing the transport of the surrogates to that of HAdV, the dominant removal mechanisms could be determined by process of elimination. The behaviour of HAdV and PRD1 phage under cycles of deionized (DI) water and high ionic strength (IS)/high pH conditions was considered, in order to test the surrogate under varying chemical conditions.

2 Enumerating Microorganism Surrogates for Groundwater Transport Studies Using Solid-Phase Cytometry

Abstract

Investigations on the pollution of groundwater with pathogenic microorganisms, e.g. tracer studies for groundwater transport, are constrained by their potential health risk. Thus, microspheres are often used in groundwater transport studies as non-hazardous surrogates for pathogenic microorganisms. Even though pathogenic microorganisms occur at low concentrations in groundwater, current detection methods of microspheres (spectrofluorimetry, flow cytometry and epifluorescence microscopy) have rather high detection limits and are unable to detect rare events. Classical microbiological enumeration methods often use a 1 ml sample volume, although including concentration steps in order to increase the sample volume is also possible. Plaque counting techniques can be quite sensitive, detecting down to one plaque per petri dish; however, if membrane filtration and microscopy are used, a low number of fields counted decreases the sensitivity of the method. It is not customary to view the whole filter manually with microscopy as it is too time consuming. Solid-phase cytometry (SPC) offers the unique capability of reliably quantifying extremely low concentrations of fluorescently labelled microorganisms or microspheres in natural waters, including groundwater, because it scans the entire filter area automatically. Until now, microspheres have been used in combination with SPC only for instrument calibration purposes and not for environmental applications. In this study the limits of the SPC methodology for its applicability to groundwater transport studies were explored. The SPC approach proved to be a highly sensitive and reliable enumeration system for microorganism surrogates down to a minimum size of 0.5 µm, in up to 500 ml of groundwater, and 0.75 µm, in up to 1 ml of turbid surface water. Hence, SPC is proposed to be a useful method for enumerating microspheres for groundwater transport studies in the laboratory, as well as in the field, when non-toxic, natural products are used.

2.1 Introduction

Scientists need a dependable method that would make it possible to predict with confidence the setback distance of a drinking water well from a potential point of contamination. Since it is not permissible to perform field tests using pathogenic microorganisms, it is necessary to predict the groundwater transport of hazardous microbes in a different way, using surrogates, such as bacteriophages (Deborde et al., 1999, Schijven et al., 2003) and synthetic microspheres, also known as beads or nanoparticles (Bales et al., 1995, Rudolph et al., 2010). Microspheres can be made from different materials; common materials are iron, silver,

latex, polystyrene and silica. For environmental field studies, silica microspheres are a preferable surrogate due to the presence of silica in natural environments. In addition to modelling colloid transport in the subsurface, microspheres can also be used to assess the surface-groundwater interaction. For example, fluorescent microspheres have been used to investigate the stream-subsurface exchange of particles in a flume in the laboratory (Arnon et al., 2010), as well as in a natural stream. Groundwater under the direct influence of surface water (referred to as GUDI or GWUDI in North America) is important when determining the vulnerability of a drinking water well to contamination from surface water.

Many different properties have an effect on colloid transport. To name a few, the surface charge of the colloid (depends on pH and ionic strength of the solution), hydrophobicity, size and shape all have an effect on how the colloid particles are transported (Schijven and Hassanizadeh, 2000). Thus, for colloid transport experiments, it is more realistic to use surrogates that are approximately the same size as the microorganisms they represent. Bacteriophages generally range in size from 0.02 to 0.20 µm and commercially available microspheres from 0.02 to 10 µm. Likewise, viruses commonly range in size from 0.02 to 0.30 µm, bacteria from 0.10 to 5 µm and protozoans from 1 to several hundred µm. In order to test the efficacy of surrogates as representatives of pathogenic microorganisms, appropriate measuring techniques are mandatory for the comparison. The constraining factor for comparing the transport of microspheres with microorganisms is the detection limit of the measuring method or apparatus. For example, bacteriophages are typically enumerated using the plaque technique, the detection limit being theoretically one plaque forming unit per petri dish, which usually contains 1 ml or less of sample, although concentration techniques are sometimes used to increase the possible sample volume (ISO, 1995). For enumeration of bacteria, Baudart et al. (2002) detected down to one *E. coli* cell on m-Endo medium, after membrane filtration of 100 ml of spiked drinking water. Quantification methods for protozoan parasites usually involve a procedure to tag the cysts or oocysts fluorescently, and then to enumerate them using a microscope. Campbell et al. (1992) enumerated over 100 *C. parvum* per filter, using sample sizes of 10 µl. Traditionally, microspheres are enumerated using spectrofluorimetry, flow cytometry or epifluorescence microscopy, with detection limits of approximately 1×10^{8}, 1×10^{2} and 5×10^{2} particles ml^{-1}, respectively, for particles that are 100 to 200 nm in diameter ((Pang et al., 2009) and citations therein). The detection limit of the enumeration method is important because the concentration of colloids affects the transport; higher influent concentrations can cause

ripening or blocking. Ripening happens when attached cells become favourable attachment sites, enhancing removal, and blocking happens when the attached cells become less favourable attachment sites, resulting in less cell retention. Bradford and Bettahar (2006) found that concentration does have an influence on attachment, but their study was limited to using influent concentrations of 10^5 to 10^7 microspheres ml^{-1} due to their enumeration method being spectrofluorimetry.

Solid-phase cytometry (SPC) is an attractive option for enumerating colloids for groundwater transport experiments in the lab and in the field because the detection limit of the method is low (theoretically 1 particle per scanned filter area; Mignon-Godefroy et al., 1997), the enumeration process is fast, and the strain on the technician is minimal, with reproducible results. The SPC detection system consists of a laser that scans the whole area of a filter onto which the target particles have been concentrated and identifies all fluorescent particles (the system aborts if memory capacity is exceeded), which are subsequently discriminated based on settings defined by the user (Mignon-Godefroy et al., 1997). In the past 15 years, several studies have shown that it is possible to detect and enumerate low numbers of labelled microorganisms in sterile and/or environmental waters with SPC (Baudart et al., 2002, Lemarchand et al., 2001, Mignon-Godefroy et al., 1997, Schauer et al., 2012) with one labelled cell detected among 10^7 to 10^8 unlabelled (non-target or artificially spiked) cells (Mignon-Godefroy et al., 1997). Reynolds et al. (1999) enumerated *Cryptosporidium parvum* oocysts using SPC (Chem*Scan*™ RDI) in three different river waters and Hijnen et al. (2005) enumerated *Cryptosporidium parvum* oocysts and *Giardia intestinalis* cysts using SPC for groundwater experiments using columns of sand and alluvial gravel. Fluorescent microspheres are commonly used for calibration of the SPC system and their detection limit has been tested in sterile water (Lemarchand et al., 2001, Lisle et al., 2004) but, up until now, the limits of detection and quantification of microspheres in environmental water samples (e.g. for groundwater transport studies like column experiments in the laboratory or outdoor in-situ experiments) via SPC has not been tested. Environmental waters differ from sterile water because they have different properties due to suspended particle and microorganism loads. For example, buoyant density affects the different settling rates of beads and bacteria during the filtration process (Lisle et al., 2004), which in turn could affect whether the particles and bacteria settle first or if they settle on top of the beads, subsequently hiding them from laser detection.

The aim of this study was thus to ascertain the limits of detection and quantification of beads via SPC in environmental waters with a special focus on groundwater. This included determining the minimum size of beads that can be detected, the extent of interference from the background matrix, and the maximum filterable volume. In addition to groundwater, surface waters with varying background matrices (bacteria and particles) that could potentially influence groundwater resources were investigated to explore the limits of the microsphere/SPC approach.

2.2 Materials and Methods

2.2.1 Sampling

Table 2.1: Water quality parameters

Measured water quality parameters and total heterotrophic bacteria (Total Bacteria) count. TOC: total organic carbon; DOC: dissolved organic carbon; TSS: total suspended solids. Electrical conductivity (EC) for AGW1[a] was not measured

Water Sample	Total Bacteria	TOC	DOC	TSS	EC	pH
	cells ml^{-1}	mg l^{-1}	mg l^{-1}	mg l^{-1}	($\mu s\ cm^{-1}$)	-
AGW1[a]	8.00×10^4	2.0	1.7	< 5	-	7.5
AGW1[b]	9.24×10^4	1.5	1.4	< 5	552	7.3
Danube	1.40×10^6	2.5	1.5	7	429	8.5
DKAS1	1.57×10^4	0.47	0.46	< 5	339	7.7
Neusiedler See	9.15×10^6	13.8	12.9	14	1600	8.7
Oberer Stinkersee	5.85×10^7	67.0	30.9	730	6400	9.6

[a] sample taken in May, 2011 for SPC tests with beads
[b] sample taken in October, 2011 for filtration volume tests

Water samples representing a wide range of background matrices from sites in eastern Austria (Table 2.1) were spiked with microspheres and tested with SPC: porous groundwater from the Lobau, a backwater system of the Danube located on the eastern border of the city of Vienna, river water from the Danube, and lake water from Neusiedler See and Oberer Stinkersee. Water from the Lobau (National Park Donau-Auen) was sampled from a groundwater well in an alluvial aquifer (AGW1) and from the left bank of the Danube between river kilometre 1910 and 1909 (Baart et al., 2010). The sample from the Neusiedler See was taken at a representative station in the centre of the lake (Kirschner et al., 2008)

and Oberer Stinkersee water was sampled from the east bank of the saltwater lake (Eiler et al., 2003), which is located within the Neusiedler See - Seewinkel National Park. All samples were taken between January and May, 2011, in clean, sterile 1 L or 2 L bottles by first rinsing the bottle with sample water and then taking the sample 30 cm below surface for all except groundwater. Samples were kept cool on ice during transport and were refrigerated at 6±2ºC in the lab until analysis. Additionally, for the determination of the maximum filtration volume possible, karstic groundwater (DKAS1) was obtained from a spring located in the Northern Calcareous Alps (approximately 100 km southwest of Vienna). This particular karstic groundwater system receives a high inflow of surface particles and bacteria during strong rainfall events as well as during snow melt (Farnleitner et al., 2005). All sterile water used for the experiments was autoclaved ion-free reverse osmosis water.

2.2.2 Environmental Parameters

Background heterotrophic bacteria were enumerated in the environmental waters using a Nikon Eclipse 80i epifluorescence microscope and the method outlined by Kirschner and Velimirov (1997). Water quality measurements were done according to DIN standards (DIN, 1987, DIN, 1997). Water temperature, electrical conductivity and pH were measured *in situ* at the time of sample collection using a WTW MultiLine P4 meter (WTW GmbH, Weilheim, Germany).

2.2.3 Particle Enumeration

Particles

Fluoresbrite™ brand of yellow-green fluorescent beads (Polysciences Inc., Warrington, PA) are commonly used for environmental studies (Harvey et al., 1989, Knappett et al., 2008, Lisle et al., 2004) due to their strong fluorescent intensity and emission/excitation spectra being similar to fluorescein (according to the manufacturer), a common dye used for cytometry and epifluorescence application. These carboxylated polystyrene spheres were purchased in various sizes (0.2, 0.5, 0.75 and 1 µm in diameter) and were diluted to desired concentrations using Polysciences Bead Solution (buffer). A ten-fold dilution series was done for each bead size to final stock solutions of 10^4, 10^3, 10^2 and 10^1 beads per ml. 100 µl of each concentration was mixed with different volumes of each environmental water sample so that the samples contained 10^3, 10^2, 10^1 or 10^0 beads per sample (not per ml).

Filterable volume

Different volumes of each sample were assayed in order to find the limit of background interference for SPC, and the optimal sample volume of each water type for practical purposes. The maximum number of allowable particles before the SPC system aborts is defined by the designated memory capacity. This criterion is subject to the total memory capacity of the particular computer being used and therefore, for these experiments, the program's default was used for the number of allowable particles in order to make the results relatively comparable.

Solid-phase cytometer

The SPC system used for this work was the Chem*Scan*™ RDI (AES Chemunex, Ivry sur Seine, France). This system has been used in the past mainly for the detection of bacteria in environmental samples (see citations in Section 1.) and is the only one which is directly connected to an epifluorescence microscope, enabling rapid visual verification of detected signals. The settings of each SPC system vary, and therefore, an optimum balance needs to be found between settings (fluorescence intensity, size etc.) that are broad enough to detect all target particles, and narrow enough such that a large number of background particles are not identified after discrimination. The discriminant settings used for the experiments in this paper are listed in the supplemental information in Appendix A (Table A1).

Filtration and mounting

The samples were filtered using a multi-fold vacuum filtration device (Pall, Port Washington, NY) onto 25 mm black polyester 0.4 µm pore size filters (AES Chemunex). For the 0.2 µm particles, it was necessary to dye 0.02 µm pore size aluminum oxide filters (Anodisc, Whatman, Billerica, MA) with Irgalan black, following the method of Hobbie et al. (1977). The stained filter or black polyester filter was then carefully placed on a support pad (AES Chemunex), which was already saturated with 100 µl of phosphate-buffered saline, prepared on the Chem*Scan*™ RDI sample holder.

Validation

Validation was done for all samples using a Nikon Eclipse 80i epifluorescence microscope, directly connected to the Chem*Scan*™ RDI system, with a 100 × magnification objective (final magnification: 1000 ×). Up to 150 events were validated per filter and all events were validated when enumeration results were less than 150. At least 3 replicates were analysed for each concentration and water sample.

2.2.4 Column Tests

To illustrate the applicability of the SPC method, column tests (similar to set-up described by Jin et al. (2000) for saturated columns) were done using the 0.5 µm beads and influent concentrations of 1.5×10^2, 1.4×10^4 and 1.8×10^6 beads ml^{-1}. The columns consisted of 30 cm long Plexiglas tubes, with an inner diameter of 7 cm, and contained quartz sand (grain size 0.4 – 0.8 mm) fully saturated with Vienna tap water (pH 8, electrical conductivity 250 µS cm^{-1}). The experiments were conducted at a Darcy velocity of 2.7 m d^{-1}, pumped upward, and effluent was collected continuously throughout the experiments.

2.2.5 Statistical Analysis

Statistical analysis (Mann-Whitney U-test, including Bonferroni correction for multiple comparisons) was performed with SPSS Statistics 17.0 software package (Chicago, IL). Results were considered statistically significant when $p < 0.05$ for dual comparisons, and < 0.01 for multiple comparisons.

2.3 Results and Discussion

2.3.1 Particle Size

The smallest particles that could be reliably detected in sterile water were 0.5 µm beads, with minimum intensities of around 200 arbitrary Chem*Scan*™ RDI fluorescent units (FU) per bead. Consequently, the minimum bead size tested in the environmental samples was 0.5 µm and the results were compared to the enumeration in sterile water for each corresponding concentration. This particle size corresponds well to the size of health related bacteria in environmental waters, like faecal indicator bacteria, for the detection of which a standard filter size of 0.45 µm is used (ISO, 2000a). If it were physically possible to manufacture beads with more molecules of fluorochrome per bead, then perhaps particles smaller than 0.5 µm could be detected by SPC. The minimum particle size detection limit of the Chem*Scan*™ RDI was not tested by Mignon-Godefroy et al. (1997), but the sensitivity of the argon laser was assayed using 1 µm polystyrene beads with 4.5×10^4 molecules of fluorochrome per bead. It was found that beads containing greater than 2000 molecules of fluorochrome could be detected. Assuming that the number of fluorochromes per bead is directly proportional to the surface area and that fluorochromes are distributed evenly over the surface area, 0.21 µm diameter beads should be detectable with SPC.

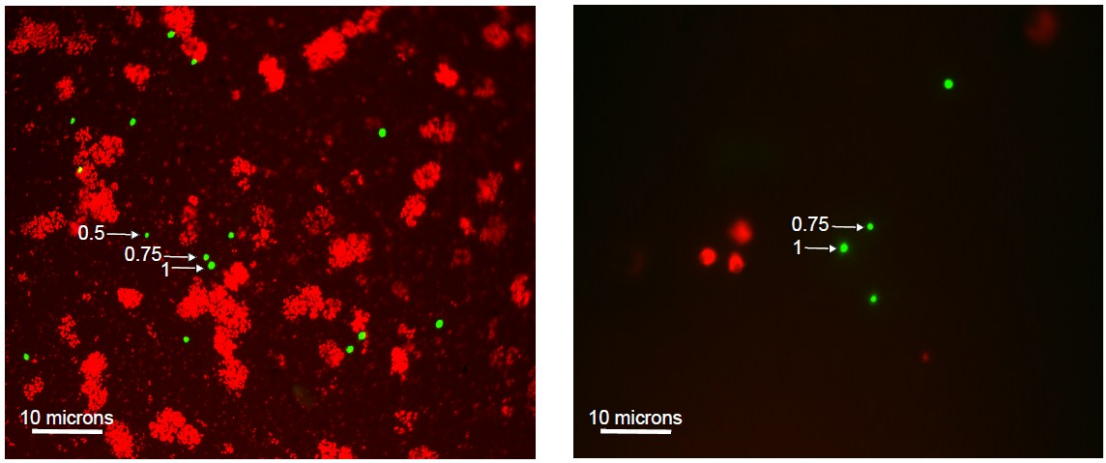

Figure 2.1: Background interference of non-target particles

Oberer Stinkersee lake water (left) and Danube river water (right) exhibit dramatic differences in the concentration of autofluorescent background particles. Background particles are red and microspheres (0.5, 0.75 and 1 µm sizes shown) are green

2.3.2 Background Interference

Bacterial cells (Lemarchand et al., 2001) or algae present in natural waters may occur in such high numbers that they mask the fluorescence of targeted particles. Furthermore, cyanobacteria or non-target particles retained on the filter can not only physically cover target cells, but can autofluoresce (Lisle et al., 2004), and could be identified as false positives. These phenomena are illustrated in Figure 2.1; the photo on the left shows the high density of background particles (coloured red) present in the Oberer Stinkersee lake water, which partially hid target particles, and the photo on the right shows spherical shaped background particles (also red) in the Danube river water, which could easily be mistaken for microspheres if it were not for the size and colour settings.

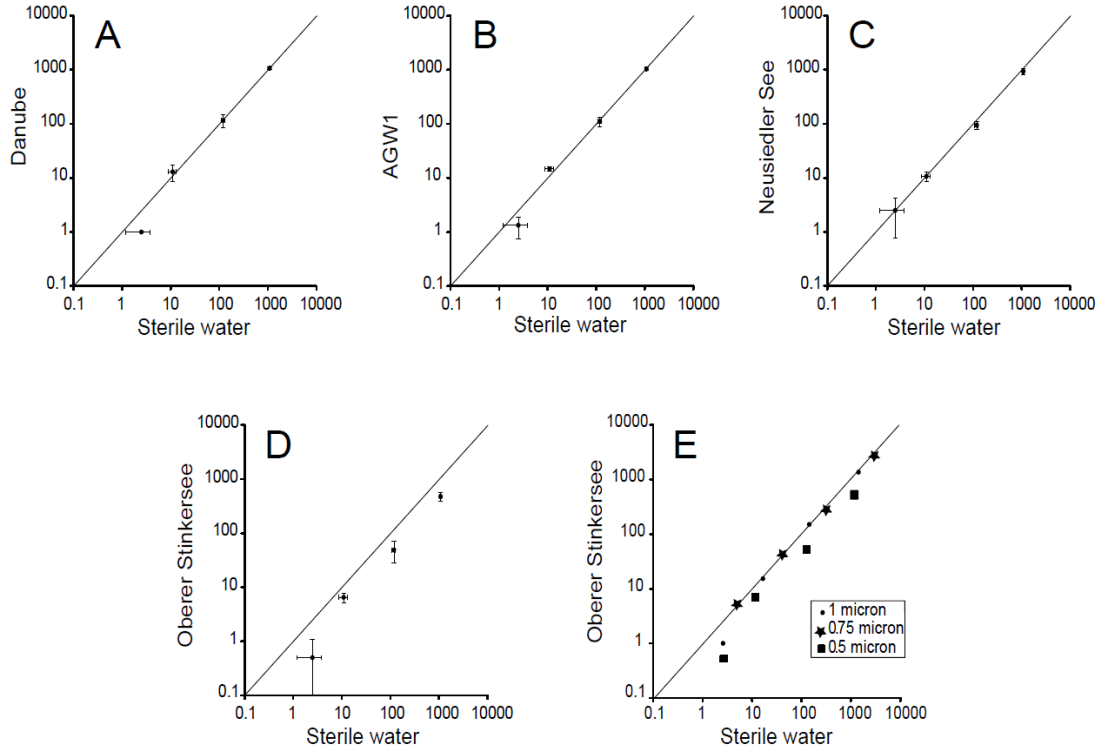

Figure 2.2: Calibration of microsphere concentrations in various background matrices

Relationship between bead enumerations (per filter) in sterile water and in Danube river water (A), AGW1 groundwater (B), Neusiedler See lake water (C), and Oberer Stinkersee lake water (D and E); data points on the 1:1 line represent results that are not negatively impacted by background matrix. Horizontal and vertical bars (graphs A through D) represent standard deviations from the mean values (shown) and replicates (n = 3 – 8). Graph E shows the relationship for beads of various sizes while graphs A through D show the results for 0.5 µm beads only

The accuracy of the number of labelled particles detected with SPC can be reduced due to the necessary narrower discrimination settings, causing some targets to be overlooked. However, by optimizing the discrimination settings and visual confirmation, this error can be minimized. The averages of the enumeration results from each environmental sample were plotted against the averages of the results in sterile water for each bead concentration tested. If there was no influence from the background matrix, results should appear on a 1:1 line. To interpret the results shown in Figure 2.2 it was assumed that the bead count in sterile water represents the true concentration and that results appearing on the 1:1 line (shown in Figure 2.2) represent high accuracy.

Results close to the 1:1 line are satisfactorily accurate, and results obviously not touching the 1:1 line are considered inaccurate (Figure 2.2, graphs D and E). It is apparent that accuracy decreases at low concentrations (to be expected) and it was also found that the detection of

the 0.5 µm beads in the Oberer Stinkersee water was inaccurate for all concentrations, probably because the high concentration of background particles covered some of the target particles. On average, the percentages of 0.5 µm beads not detectable in the Oberer Stinkersee water were 80%, 41%, 59%, and 56% for 10^0, 10^1, 10^2, and 10^3 beads per filter, respectively. Furthermore, at low concentrations, precision decreases on both axes (shown by large standard deviations) due to unavoidable processing error. Table A2, in Appendix A, shows calculated variation coefficients (indicating precision of collected data) and p-values from the Mann-Whitney U-test, which tests for significant differences between the measurement of beads in environmental water and sterile water.

2.3.3 Detection and Quantification Limits

In practice, there are two kinds of detection limits that have to be considered: method detection limit (MDL), and sample limit of detection (SLOD). MDL is the theoretical detection limit under ideal laboratory conditions for a given method, while SLOD is the estimated detection limit associated with a particular sample (considering sample volume and background matrix) (Domingo et al., 2007). Likewise, there are two kinds of quantification limits: method quantification limit (MQL), and sample limit of quantification (SLOQ). Included in the SLOD and SLOQ are operational variability and intrinsic variability. Operational variability is the uncertainty of the results due to the technical steps of the analytical method and intrinsic variability is the uncertainty inherent in the random distribution of particles (based on a Poisson distribution) (ISO, 2000b). The MDL and MQL of SPC are predefined as 1 particle per filter, because the SPC system is able to detect and enumerate a single target particle on the whole filter surface (Mignon-Godefroy et al., 1997). SLOD and SLOQ depend on the volume of sample analysed and interference from background matrix and were determined by spiking beads in different environmental waters with different water quality characteristics.

The volume of each sample tested depended on the amount of background material (suspended particles or microorganisms) that caused the SPC to abort, in other words, when the memory capacity is exceeded, as defined by the software, due to too much data. The SPC system aborted when 4 ml of the Danube water was filtered, containing 5.6×10^6 bacterial cells and 28 µg TSS (total suspended solids, calculated from Table 2.1), which produced excess background fluorescence; therefore, only 3 ml was used for the enumeration tests. One ml of Neusiedler See and Oberer Stinkersee water could be analysed

successfully, even though the samples contained 9.2×10^6 and 5.9×10^7 cells, as well as 14 and 730 µg TSS, respectively (Table 2.1). AGW1 water did not cause the SPC system to abort after filtering 50 ml, but the system aborted when 100 ml was used. It was decided that 15 ml of the AGW1 water was the appropriate volume to use for the experiments, considering that this is often the practical volume that is collected per sample during groundwater column experiments in the laboratory (Jin et al., 2000). The sample volume limit of a karstic groundwater sample (DKAS2) was determined for comparison. 500 ml of the clear karstic water could be filtered and tested with SPC; the SPC system aborted when 1 L was filtered.

The maximum volume of sample water that can be processed for microorganism enumeration is important when determining the SLOD and SLOQ (e.g. 1 particle detected in 1 L represents a 1,000 fold lower detection limit than 1 particle in 1 ml). For this work, the SLOD is defined to be 3 particles per maximally filterable volume, according to a Poisson distribution of randomly distributed particles (intrinsic variability), assuming a 95 % confidence interval (ISO, 2000b). SLOQ is defined as 4 particles per volume, based on an acceptable relative precision of 50 % (coefficient of variation = 0.50), which seems to be reasonable in microbiology, according to ISO 8199:2005 (ISO, 2005). If one requires a higher relative precision of 30% (CV = 0.30), then SLOQ would be 11 particles per volume (ISO, 2000b). Experiments performed in the same laboratory (Schauer et al., 2012) showed that as few as 13 microspheres could be reliably quantified with a variation coefficient of 26.5 %, following the protocol outlined in ISO 13843:2000 (ISO, 2000b).

Table 2.2: Limits of detection and quantification

Limits of detection and quantification for enumeration of fluorescent microspheres of minimum enumerable sizes (0.5 µm for all waters except for Oberer Stinkersee) by solid-phase cytometry. MDL: method detection limit; SLOD: sample limit of detection; 3 is used as the minimum number of particles to be detectable in a water sample according to ISO 13843:2000 (ISO, 2000b); MQL: method quantification limit; SLOQ: sample limit of quantification; 4 is used as the minimum number of particles to be quantifiable in a water sample according to ISO 8199:2005 (ISO, 2005). A more detailed explanation about limits of detection and quantification can be found in the discussion section

Water Sample	Filterable volume	MDL	SLOD	MQL	SLOQ
	ml	beads filter^{-1}	beads ml^{-1}	beads filter^{-1}	beads ml^{-1}
AGW1	50	1	0.06 (3 / 50 ml)	1	0.08 (4 / 50 ml)
Danube	3	1	1 (3 / 3 ml)	1	1.3 (4 / 3 ml)
DKAS1	500	1	0.006 (3 / 500 ml)	1	0.008 (4 / 500 ml)
Neusiedler See	1	1	3 (3 / 1 ml)	1	4 (4 / 1 ml)
Oberer Stinkersee	1	1	3 (3 / 1 ml)	1	4 (4 / 1 ml)

From the maximum filterable volumes, the SLOD and SLOQ were calculated (Table 2.2). Because not more than 3 ml of river water from the Danube could be filtered and processed, the SLOD for river water is 1 bead per ml (3 beads per 3 ml; Table 2.2). One ml of Neusiedler See and Oberer Stinkersee was the maximum volume that could be successfully analysed, resulting in an SLOD of 3 beads per ml for highly turbid aquatic environments. Up to 500 ml of DKAS2 water and 50 ml of AGW1 groundwater could be filtered before the SPC system aborted, resulting in SLODs of 0.006 (3 per 500 ml) and 0.06 (3 per 50 ml), respectively (Table 2.2). The SLODs and SLOQs are improved accordingly as the volume filtered is increased (Table 2.2); however, 50 ml is a common sample size for microorganism tracer tests in the field (Deborde et al., 1999) and 15 ml is common for laboratory experiments (as mentioned above).

2.3.4 Column Tests

In order to demonstrate the innovative aspect and practicality of the method, column tests were performed using various influent concentrations, ranging in magnitude from 10^2 to 10^6 beads ml^{-1} (Figure 2.3). Since traditional enumeration methods, such as spectrofluorimetry, flow cytometry and epifluorescence microscopy, are not able to enumerate the range of effluent concentrations observed (10^0 to 10^4 beads filter^{-1}), such a comparison of influent

concentrations would not have been possible without the use of SPC. For influent concentrations of 10^2, 10^4 and 10^6 beads ml^{-1}, sample volumes were 114 ml, 14 ml, and 1 ml and SLOQ values were 0.03, 0.3 and 4, respectively.

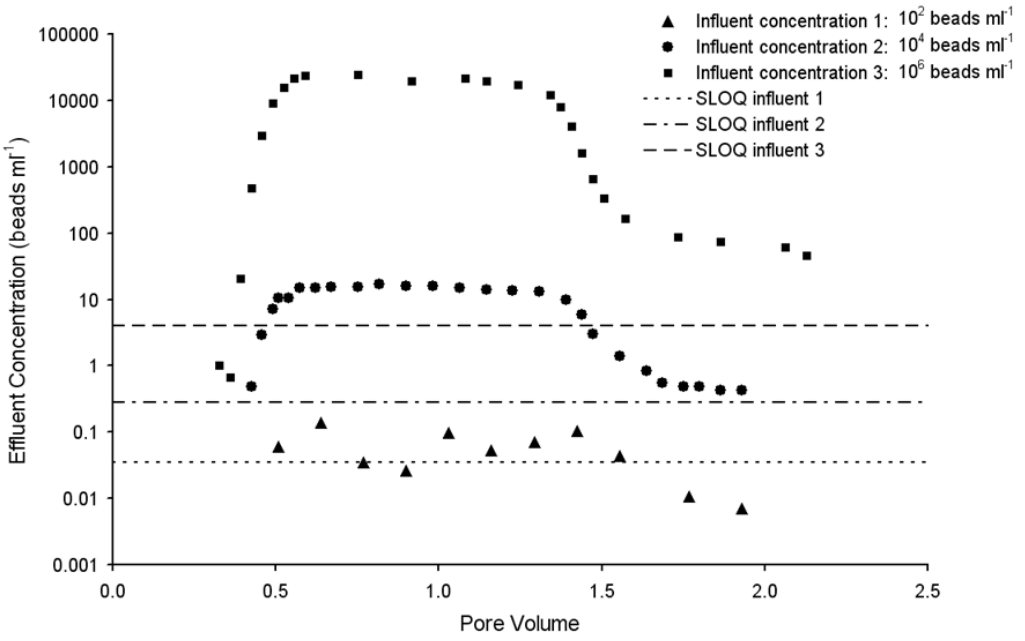

Figure 2.3: Column test breakthrough curves

Breakthrough curves and SLOQ values of column tests with influent concentrations of 10^2, 10^4 and 10^6 beads ml^{-1}. Data points under the SLOQ line are not considered quantifiable. The x-axis describes the cumulative volume of effluent water relative to the total water content the column (pore volume).

2.4 Conclusions

An obvious advantage of using SPC with a surrogate tracer like microspheres is that microspheres are much easier to work with compared to microorganisms; the samples do not have to be kept on ice, groundwater column tests in the lab can be performed at room temperature, no special precautions or training concerning pathogens are required and sterile conditions are not mandatory. If non-toxic, natural materials are used, such as silica, application in field studies is also possible. Another advantage of using SPC with microspheres is that it is both fast and relatively easy compared to cultivation methods.

Lastly, the most important advantage of using SPC for groundwater transport experiments is that the detection limit is much lower than that of the traditional methods for enumerating microspheres using spectrofluorimetry, flow cytometry or epifluorescence microscopy. The method allows enumeration of target particles down to a minimum size of 0.5 µm in porous and fractured groundwater, for sample volumes of up to 500 ml. In addition, this methodology can also be used for testing the influence of highly turbid surface water (or even wastewater) on a respective groundwater resource, when particles down to a minimum size of 0.75 µm and sample volumes of up to 1 ml are used. We thus propose that SPC is a superior method that can be used to quickly detect and enumerate low numbers of surrogate particles in diverse water matrices.

3 Biotin- and Glycoprotein-Coated Microspheres as Surrogates for Studying Filtration Removal of *Cryptosporidium parvum* in Granular Limestone Aquifer Media

Abstract

Cryptosporidium is a waterborne protozoa of great health concern. Many studies have attempted to find appropriate surrogates for assessing *Cryptosporidium* filtration removal in porous media. In this study, the filtration of *Cryptosporidium parvum*, biotin- and glycoprotein-coated carboxylated polystyrene microspheres (CPM) was evaluated in granular limestone media. Column (10 cm long and 1.5 cm internal diameter) experiments were carried out with core material taken from a managed aquifer recharge site in Adelaide, Australia. For the experiments with injection of a single type of particle, a total removal of the oocysts and glycoprotein-coated CPM, 4.6-6.3 $\log_{10}$ reduction of biotin-coated CPM and 2.6 $\log_{10}$ reduction of unmodified CPM was observed. When two different types of particles were simultaneously injected, glycoprotein-coated CPM showed 5.3 $\log_{10}$ reduction while the uncoated CPM displayed 3.7 $\log_{10}$ reduction, probably due to particle-particle interactions. The results confirm that glycoprotein-coated CPM are the most accurate surrogates for *C. parvum*; biotin-coated CPM are slightly more conservative while unmodified CPM are markedly over-conservative for predicting *C. parvum* removal in granular limestone media. A total removal of *C. parvum* observed in this study suggests that granular limestone media is very effective in filtration removal of *C. parvum* and could potentially be used for the pre-treatment of drinking water and aquifer storage recovery of recycled water.

3.1 Introduction

Waterborne cryptosporidiosis is mainly caused by *Cryptosporidium parvum* and *Cryptosporidium hominis* in humans (Plutzer and Karanis, 2009). *Cryptosporidium* could be found in water contaminated with infected human or animal feces, has a low infectious dose and ingestion of fewer than 10 oocysts can lead to infection (WHO, 2004). *Cryptosporidium spp.* oocysts are sometimes detected in drinking water supplies (Lechevallier et al., 1991) and in potable groundwater (Willocks et al., 1998), causing disease outbreaks. For example, in the 1993 cryptosporidiosis outbreak in Milwaukee (USA), about 400,000 people were infected and more than 100 people died after contamination of drinking water by *C. parvum* (Mackenzie et al., 1994). More recently, a waterborne outbreak of cryptosporidiosis in Östersund, Sweden that infected 27,000 people in 2010, was caused by *C. hominis* (Widerstrom et al., 2014).

C. parvum can survive in surface-water and groundwater for a long period of time (Sidhu et al., 2010) and is resistant to chemical disinfection, like chlorination (Carpenter et al., 1999) and ozonation (Korich et al., 1990), due to its thick oocyst wall. Ultraviolet irradiation techniques with low and medium pressure lamps have been found to be effective at inactivating *C. parvum* (Clancy et al., 2000). However, the efficacy of UV irradiation as well as that of chemical disinfection is hampered by turbidity in the water. Thus, filtration is often used as an essential primary step for drinking water treatment in the course of a multi-barrier treatment system because it is effective and cost efficient.

Being extremely infectious and highly resistant to chlorination, *C. parvum* is often used in risk analysis of drinking-water supplies (Page et al., 2010). However, due to the high analytical costs involved, *C. parvum* is not routinely monitored in water treatment. Traditionally, water turbidity is used as a performance measure for *C. parvum* removal in water supplies but turbidity does not correlate well with the presence of *C. parvum* (Emelko et al., 2005). *Clostridium perfringens*, which is included in the European Drinking Water Directive (EU, 1998), is a potential surrogate for *C. parvum* due to its low inactivation rate (Schijven et al., 2003), even though *Clostridium perfringens* is five times smaller and may have different filtration characteristics.

Some researchers have used unmodified carboxylated polystyrene microspheres (CPM) as a surrogate for studying *C. parvum* filtration (Dai and Hozalski, 2003, Harvey et al., 2008, Mohanram et al., 2010), but the results are generally unsatisfactory (Harvey et al., 2011). Although CPM could mimic the size, buoyant density and spherical shape of the oocysts, unmodified CPM are significantly more negatively charged than oocysts (Dai and Hozalski, 2003, Pang et al., 2012). In addition, unlike oocysts, unmodified CPM lack a macromolecular structure, which makes them poor surrogates for *C. parvum* (Kuznar and Elimelech, 2005, Kuznar and Elimelech, 2006).

Surface characteristics play an important role in particle retention and transport in porous media. Thus, for CPM to be better surrogates, their surface properties need to be modified. A recent study by Pang et al. (2012) has demonstrated that glycoprotein- and biotin-coated CPM are superior to unmodified CPM in predicting filtration removal of non-inactivated *C. parvum* oocysts, showing the same $\log_{10}$ reduction in concentration. These biomolecules have similar surface charge to that of *C. parvum* with an isoelectric point pH≈2 (Pang et al.,

2012) and glycoprotein is the major type of protein that *C. parvum* produces on its cell surface (Waldron et al., 2009). However, only transport in alluvial sand media was validated in their study and it is unknown whether these modified CPM are also useful in predicting *C. parvum* filtration in other aquifer media with different chemical properties.

Limestone aquifers supply potable groundwater in many parts of the world, and can be very vulnerable to fecal contamination (Stadler et al., 2008), making catchment and water quality management an essential requirement (Reischer et al., 2011) to guarantee drinking water quality. Many managed aquifer recharge (MAR) sites are also developed in limestone aquifers, e.g., in Australia and Mexico (Dillon et al., 2008), and in the southeast United States (Mirecki et al., 1998). Thus investigating *C. parvum* filtration in limestone aquifer media has important implications to aquifer management and risk analysis of potable groundwater. Harvey et al. (2008) previously investigated formalin-inactivated *C. parvum* and CPM (1.6, 2.9 and 4.9 μm in size) in intact cores of fractured limestone. In their study, CPM significantly over-predicted *C. parvum* removal with a fractional recovery of 19% for *C. parvum*, 3% for 4.9 μm CPM and 5% for 2.9 μm CPM. In this study, the aim was to investigate the usefulness of glycoprotein- and biotin-coated CPM in predicting filtration removal of *C. parvum* in granular limestone aquifer media, and to evaluate the efficacy of granular limestone media for the removal of *C. parvum* by filtration.

3.2 Materials and Methods

3.2.1 Aquifer Material

The porous media used in the column experiments was sourced from a tertiary limestone aquifer at the MAR site in Parafield Gardens, Adelaide, Australia (Dillon et al., 2008). Figure 3.1 shows a schematic of the treatment steps for this MAR site which recycles urban stormwater runoff. Core samples were taken from a depth of 170−180 m below ground surface at 34.793°S, 138.622°E. The aquifer material was granular and heterogeneous in size containing some consolidated chunks. The coarse and fine fractions were sieved out so that the grains of 0.25−2.4 mm in size (approximately 20% of the original material) were used in the column experiments (mean diameter d_{50} = 0.55 mm, uniformity d_{60}/d_{10} = 1.94). The material was then rinsed with deionized water and oven dried at 80°C overnight. X-ray diffraction analysis showed that the aquifer material consisted mainly of calcite (63%) and

quartz (31.3%). Other minerals included microcline (1.6%), aragonite (1.5%), albite (0.7%), goethite (0.6%), pyrite (0.5%), ankerite (0.4%), and hematite (0.3%).

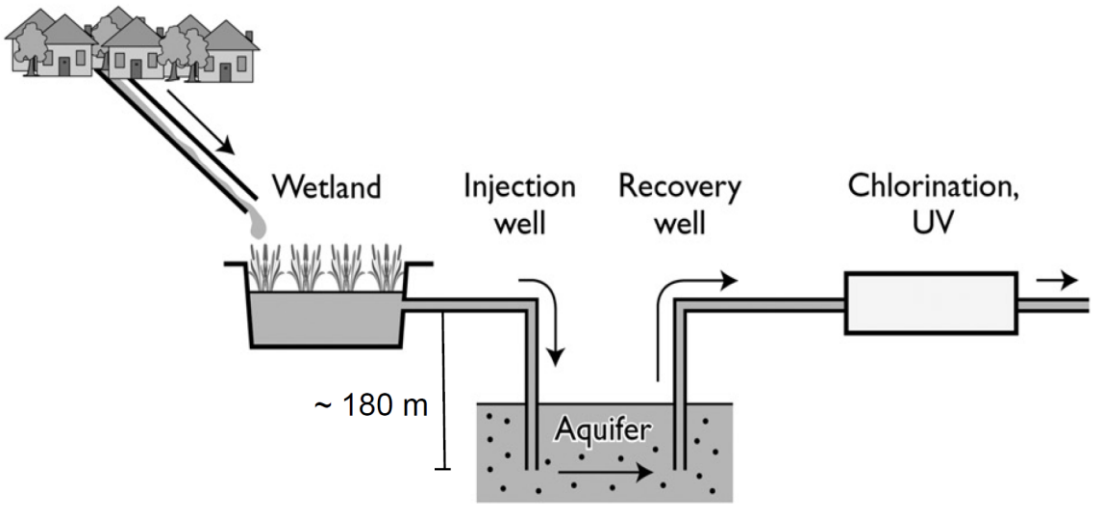

Figure 3.1: Managed aquifer recharge

Managed aquifer recharge (MAR) treatment steps for water recycling of urban stormwater runoff at Parafield Gardens, Southern Australia (modified from Page et al. (Page et al., 2010)).

3.2.2 Colloidal Particles

C. parvum oocysts are spherical or oval, 3.9−5.9 µm in diameter (Medema et al., 1998), and have an isoelectric point of pH=2.0−3.3 (Butkus et al., 2003, Hsu and Huang, 2002, Pang et al., 2012). Two strains of oocysts were used in the experiments. These included fresh human fecal samples from anonymous donors, obtained from the PathWest Laboratory Medicine, WA, Australia from already infected individuals, and the "Iowa" *C. parvum* genotype 2 isolate from Sterling Parasitology Laboratory (University of Arizona, Tucson).

The stock of the human strains was purified by a delipidation step in diethyl ether followed by sucrose flotation using a method described in Gobet and Toze (2001). The purified stock was then stored at 4°C in diethylpyrocarbonate-treated phosphate-buffer saline and used within 4 months of receiving it. Immediately before making the injection solution, the stock was exposed to a germicidal lamp (UV light at a wavelength less than 320 nm in class 2 Biohazard cabinet fitted with 20WTUV light) for 30 min to reduce its infectivity.

The stock of the animal strain was purified using discontinuous sucrose gradients and cesium chloride centrifugation gradients. Oocysts were stored in antibiotic solution (0.01% Tween 20, 100 U ml^{-1} of penicillin and 0.1 mg ml^{-1} of gentamicin) at 4°C and used within 3 months of

receiving it. The stock suspension of oocysts was diluted with 10 mM NaCl, buffered to a pH of 8.0, to a concentration of 1 x 10^6 oocysts ml^{-1}. Portions of 25 ml of the working suspension were exposed in sterile petri dishes under permanent stirring in a standard laboratory irradiation apparatus (low pressure lamps) as described in detail elsewhere (Sommer et al., 2001). The UV-254 nm fluence applied was 800 J m^{-2} UV-254 nm. The transmittance of the oocyst suspension (70% in 10 mm) was taken into consideration for the fluence calculation.

To mimic the size, spherical shape, and density of the oocysts, 4.5 µm CPM were purchased from Polysciences Inc. (Warrington, USA). The CPM were fluorescent yellow-green (YG), yellow-orange (YO), and bright-blue (BB), respectively. It was initially intended to inject the three types of CPM together. Thus, the CPM were purchased in three different colors in order to differentiate them for analysis. The YO CPM were coated with α1-acid glycoprotein (Sigma-Aldrich, St. Louis, USA) and the BB CPM were coated with amine-containing biotin (Thermo Fisher Scientific Inc., Rockford, USA), following the conjugation of biomolecules method described in Pang et al. (2012).

The zeta potentials (ζ's) of the particle solutions were measured using electrophoretic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Particle concentrations used for the measurements were 10^4 oocysts ml^{-1} and 10^6 microspheres ml^{-1}. The low concentration of oocysts used was constrained by the initial concentration of oocysts in the stock solution. A background electrolyte of 10 mM NaCl, pH=8.0 ± 0.2 was consistently used in the study. The zeta potential measurements were carried out in triplicate.

3.2.3 Column Experiments

Glass chromatography columns (10 cm long and 1.5 cm internal diameter) were packed under saturated conditions with the aquifer material. In each column, the same amount of water and dry weight of aquifer material were used so that it gave an effective porosity of 0.56 (±0.02) and a dry bulk density of 1.01 (±0.06) g cm^{-3}. A Darcy flow rate of 1.21 (± 0.15) m d^{-1}, mimicking the typical flow rate measured in-situ, was applied from the top of the column using a peristaltic pump (Masterflex, Vernon Hills, IL). The background electrolyte of 10 mM NaCl at pH 8.1±0.3 (buffered with $NaHCO_3$) was used throughout the experiments to establish an equilibrium with aquifer material containing predominantly carbonate minerals. Experiments were conducted at room temperature (22−23 °C), which was similar to the

groundwater temperature (23°C) at a depth of 160−180 m (Dillon et al., 2008) at the field site where the aquifer material was obtained.

To characterize hydraulic conductivity and dispersivity of the porous media in the packed columns, an experiment was first conducted using 1 mM sodium nitrate ($NaNO_3$) as a conservative tracer. The concentration of NO_3^- in the column effluent was measured with a UV-Vis spectrophotometer (Cary Series UV-Vis, Agilent Technologies, Santa Clara, CA) at a wavelength of 210 nm.

For the experiments with the particles, columns were first flushed for at least 18 pore-volumes using the background electrolyte and 4 pore-volumes of tracer solution were injected. Test runs were done using one type of particles at a time and columns were repacked with fresh material for each test run with an exception in one experiment, when uncoated and glycoprotein-coated CPM were injected together. Whenever possible, column tests were done in duplicate. Influent concentrations of the colloids ranged from 10^4 to 10^7 particles ml^{-1}. The influent concentrations of the coated CPM and the oocysts were relatively lower due to particle loss during the coating procedure and low initial oocyst concentration in the stock.

3.2.4 Enumeration of Particles

Initially, enumeration of the human strain oocysts was attempted with epifluorescence microscopy using the dye staining method with 4',6diamidino-2-phenylindole (DAPI) and propidium iodide, as outlined by Campbell et al. (1992). However, no typical oocysts could be identified from the microscopic analysis, even though positive controls yielded quantifiable results. Therefore, another experiment was carried out using animal strain oocysts and samples were enumerated using a more sensitive enumeration method that is appropriate for very low concentrations.

The animal strain oocysts, uncoated YG and glycoprotein-coated YO CPM were enumerated using solid-phase cytometry (Chem*Scan*® RDI, AES Chemunex, bioMérieux, Marcy-l'Étoile, France), according to the method outlined in Stevenson et al. (2014), which allows a low sample limit of detection (SLOD) of 3 particles / filtered volume. *C. parvum* were stained with *EasyStain*™ antibody (BTF Pty. Ltd., bioMérieux, North Ryde, Australia), following the instructions provided by the manufacturer. The calibration curve (Figure A1) for

enumeration of the oocysts, and more detailed information about the dying procedure, can be found in Appendix A. Calibration curves for the microspheres in different water matrices can be found in Stevenson et al. (2014). To differentiate between the YG and the YO CPM, different photomultiplier tubes (PMT's) were used. The green PMT detects an emission wavelength of 500–530 nm and the yellow/orange PMT detects a wavelength of 540–570 nm. As the emission and excitation of BB CPM are outside the range of the solid-phase cytometry, the BB CPM were enumerated using a Nikon Eclipse 8000 epifluorescence microscope. Montemayor et al. (2007) found that solid-phase cytometry and epifluorescence microscope produce equivalent results. The microscope was equipped with an automatic stage which enabled scanning of the whole filtration area and the same SLOD as for solid-phase cytometry, which scans the entire filter area with a laser. All samples were filtered onto 25-mm black polyester 0.4-µm pore size filters (bioMérieux) before the enumeration. Three replicates were analyzed for each sample and the filtered sample volume was 1 ml; thus, the SLOD for all samples analyzed was 3 particles ml^{-1} (Stevenson et al., 2014).

3.2.5 Data Analysis

The filtration efficiency of the particles was described using peak breakthrough attenuation [$\log_{10}(C_{max}/C_o)$], relative mass recovery (*RB*) and collision efficiency (α). C_{max} and C_0 are the peak effluent concentration and input solution concentration, respectively. RB was estimated by integrating the area under the breakthrough curve (BTC) of the colloid and normalizing to that of the conservative tracer nitrate. The collision efficiency, α is determined from the formula (Pieper et al., 1997):

$$\alpha = \frac{d\left[\left(1 - 2\frac{\lambda}{x}\ln(RB)\right)^2 - 1\right]}{6(1-\theta)\eta\lambda}$$

where d is the mean grain size of the sand, λ is the dispersivity, x is the transport distance, ϑ is the effective porosity and η is the single-collector efficiency. The η and α values were calculated based on the equations of Tufenkji and Elimelech (2004). Hamaker constants of 1.00×10^{-20} J for glass-water-polystyrene (Elimelech and Omelia, 1990) and 6.50×10^{-21} J for oocysts (Kuznar and Elimelech, 2005) were used. A buoyant density of 1.05 g cm^{-3} was applied to represent the density of the CPM (Polysciences Inc.) and the oocysts (Medema et al., 1998). Darcy velocity (U) and λ are required in the calculations of η and α. The measured

flow rates were used for the *U* values, while λ was optimized by simulating the nitrate data using the CXTFIT model.

3.3 Results and Discussion

3.3.1 Surface Charge

The surface charge of the human UV treated *C. parvum* measured in this study (ζ = -35.3 mV ± 1.3) was similar to the ζ values of viable *C. parvum* (ζ = -37 to -42 mV in $NaClO_4$ at pH=7.0, electrical conductivity = 0 − 2200 µS cm^{-1}) reported by Hsu and Huang (2002). Compared to the *Cryptosporidium* oocysts, the unmodified YG CPM were markedly more negatively charged (ζ = -107.0 mV ± 6.1), which is similar to what was found in Pang et al. (2012) for unmodified BB CPM (ζ = -111 mV in 1 mM NaCl pH=7). In contrast, the glycoprotein- and biotin-coated CPM mimicked the surface charge of the oocysts relatively well. Although glycoprotein and biotin have a similar surface charge (Pang et al., 2012), the charge of glycoprotein-coated YO CPM in this study (ζ = -13.3 mV ± 2.1) was lower than that of biotin-coated BB CPM (ζ = -37.3 mV ± 0.9). This is most likely due to the fact that prior to coating, the unmodified YO CPM were much less charged (e.g., ζ = -44.6 mV in 2 mM NaCl at pH=7) than the unmodified BB CPM (-111 mV as mentioned above), resulting in a lower charge after coating. For the same reason, glycoprotein-coated YO CPM synthesized in this study were less charged compared to the glycoprotein-coated BB CPM synthesized in the study by Pang et al. (2012). The reason for coating YO CPM with glycoprotein instead of BB CPM was that the YO CPM could be counted using solid-phase cytometry but the BB CPM had to be manually counted using microscopy, as mentioned previously.

Table 3.1: Summary of column experiments

Tracer	Ex.	Run	C_o (particles ml^{-1})	Note	U (cm min^{-1})	RB	η	α	T_o (min)	pH_{in}	pH_{out}	$\log_{10}(C_{max}/C_0)$
Nitrate		1	1 mM (85 mg L^{-1})		0.07	9.23×10^{-1}	-	-	290	7.85	8.24	0.0
C. parvum - human strain		1	1.00×10^{5}	[a]	0.08	0.00×10^{0} [c]	2.62×10^{-2}	[c]	224	7.78	8.23	[c]
C. parvum - animal strain		1	9.30×10^{5}	[b]	0.09	0.00×10^{0} [c]	2.45×10^{-2}	[c]	258	7.99	7.91	[c]
Glycoprotein-coated YO CPM in mix	T2A	1	1.60×10^{6}	[b]	0.09	1.30×10^{-5}	2.57×10^{-2}	4.16	220	8.21	8.33	-5.2
Glycoprotein-coated YO CPM	T6	1	6.30×10^{4}	[b]	0.08	0.00×10^{0} [c]	3.06×10^{-2}	[c]	288	7.92	8.42	[c]
Glycoprotein-coated YO CPM	T7	2	3.30×10^{5}	[b]	0.11	0.00×10^{0} [c]	2.16×10^{-2}	[c]	211	7.86	8.07	[c]
Biotin-coated BB CPM	T8	1	7.80×10^{6}	[a]	0.08	2.53×10^{-7}	2.75×10^{-2}	5.41	250	8.03	-	-6.3
Biotin-coated BB CPM	T11	2	6.50×10^{5}	[a]	0.09	3.71×10^{-5}	2.66×10^{-2}	3.63	250	7.84	8.09	-4.6
Uncoated YG CPM in mix	T2A	1	1.60×10^{7}	[b]	0.09	2.40×10^{-4}	2.57×10^{-2}	3.02	220	8.21	8.33	-3.7
Uncoated YG CPM	T3	1	8.10×10^{6}	[b]	0.07	3.02×10^{-3}	3.18×10^{-2}	1.67	260	8.10	8.46	-2.6
Uncoated YG CPM	T9	2	3.40×10^{7}	[b]	0.08	1.56×10^{-3}	2.84×10^{-2}	2.09	320	7.89	7.99	-2.6

[a] counted by epifluorescence microscopy, [b] counted by solid-phase cytometry, [c] breakthrough below sample limit of detection.

C_o - injection concentration, U - measured Darcy flow rate, RB - relative mass recovery, η - single collector factor, α - collision coefficient, T_o - pulse duration, pH_{in} - influent pH, pH_{out} - effluent pH, C_{max} - peak concentration, mix - simultaneous injection of uncoated and glycoprotein-coated microspheres.

3.3.2 Filtration Efficiency

Peak breakthrough attenuation [$\log_{10}(C_{max}/C_o)$], mass recovery (*RB*) and collision efficiency (α) (Table 3.1) were calculated for the unmodified CPM and the modified CPM (excluding the glycoprotein-coated CPM injected alone). The oocysts and the glycoprotein-coated CPM (injected alone) exhibited no breakthrough, and therefore, $\log_{10}(C_{max}/C_o)$, *RB* and α could not be calculated. If the SLOD is used as C_{max}, the oocysts would have a $\log_{10}(C_{max}/C_o)$ of -4.5 to -5.5 and the glycoprotein-coated CPM -4.3 to -5.0. Calculated in this way, log-removal of the oocysts is similar to that of the protein-coated CPM's, although for breakthrough curves that do not attain steady state, the applicability of C_{max} is debatable. Interestingly, when the uncoated and glycoprotein-coated CPM were injected together (mix), the concentrations and filtration efficiency ($\log_{10}(C_{max}/C_o)$, *RB*) of the uncoated CPM were reduced by one order of magnitude compared to when they were injected alone (Figure 3.2, Table 3.1). This is probably due to enhanced particle-particle collision, resulting in clumping, and therefore, a stronger straining effect. The breakthrough of the glycoprotein-coated CPM was below SLOD when injected alone. Presumably, when injected together, the less negatively charged glycoprotein-coated CPM attached to the more negatively charged unmodified CPM and were carried along.

The α values calculated were > 1, which is not unusual for material that is very angular in nature, and similar to those found for granular activated carbon (Paramonova et al., 2006), showing that the material is very effective at removing colloids. Low α values result in low removal, and conversely, high α values represent high removal. Theoretically, α should be between 0 and 1 because it is a ratio of the successful attachment to the number of collisions; there cannot be more attachment than there are collisions. However, in fine and/or angular material an α value > 1 may be due to removal of colloids from straining and not necessarily attachment. Even if this is the case, it is still a useful parameter for relative comparison.

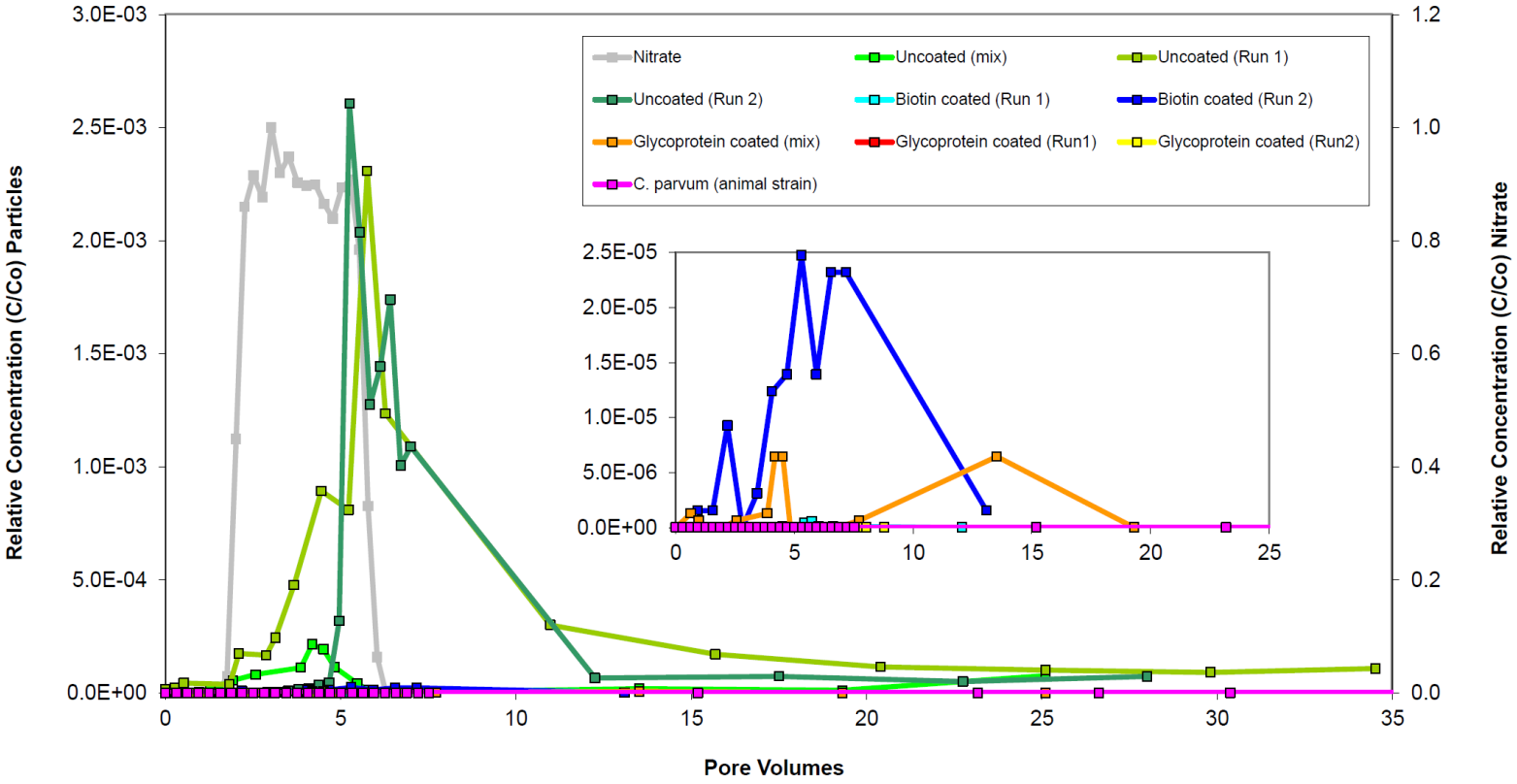

Figure 3.2: Breakthrough curves of microspheres and *C. parvum*

Relative concentrations of nitrate, *C. parvum* oocysts, unmodified and modified CPM with biotin and glycoprotein in column experiments with granular limestone aquifer media in 10 mM NaCl, pH=8. Inset graph with the expanded scale shows modified CPM and the oocysts.

Figure 3.2 demonstrates that the unmodified CPM over-predicted oocysts' concentrations in flow-through column experiments, while concentrations of the oocysts and modified CPM were on the same order of magnitude (minimal to no breakthrough at all). This finding is consistent with the study of Pang et al. (2012). The plausible explanation is that there was a greater tendency of electrostatic repulsion between the aquifer media and the more negatively charged unmodified CPM than between the aquifer media and the less negatively charged oocysts or modified CPM, as suggested by their ζ values. The results suggest that the attachment tendency of particles to the aquifer media (Table 3.1) is related to the overall surface potential (ζ value) of particles. The most negatively charged particles (YG uncoated CPM) displayed the least attachment and lowest removal, while the least negatively charged particles (YO glycoprotein-coated CPM) showed the highest attachment and the most removal. Similar to what was found in Pang et al. (2012), biotin-coated CPM slightly over-predicted oocyst concentrations, and thus, are more conservative than glycoprotein-coated CPM. Column test Run 1 with the biotin-coated CPM had lower breakthrough than Run 2. This may have been due to ripening, a build-up of colloids encouraging more attachment, caused by the higher influent concentration used for Run 1 (Bradford and Bettahar, 2006).

The size ratio of oocysts or CPM to sand grains (ϕ/d_{50}) was 0.9% (where ϕ represents the diameter of the colloid), which is above the threshold of 0.5% for straining to occur (Bradford and Bettahar, 2005). Independent of the particle to grain size ratio, the shape irregularity of the porous media may also cause straining due to smaller pore spaces being present after packing irregularly shaped media (Tufenkji et al., 2004). Upon visual examination of the porous media used in this study, the material is highly irregular in shape. Thus, straining may have played an important role in the removal of the oocysts and CPM in the aquifer media investigated here. This is reflected in their extremely low mass recovery (Table 3.1) indicating that irreversible attachment (including straining) was more dominant than reversible attachment during the experiments. Some "irreversible" sites may be due to the period of the experiment (< 30 pore-volumes) and, with a continuous flush or a change in solution chemistry, the previously strongly attached particles may detach from the porous media. For example, Harter et al. (2000) and Mohanram et al. (2010) found that initial oocyst attachment is reversible, even in soils with high iron oxide content (Mohanram et al., 2010). However, the experiments were not run long enough or change the solution chemistry to see a detachment of the oocysts and the surrogates, although the uncoated CPM exhibited

some degree of reversible attachment as suggested from their tailings in the breakthrough curves.

In this study it was observed that the recovery of unmodified CPM in granular limestone material was markedly higher than that of *C. parvum*, which demonstrated total removal (zero recovery). However, the opposite was observed in the column experiment (10 mM artificial groundwater, pH=8, flow rate 23 m d^{-1}) of Harvey et al. (2008) with intact karst limestone core (16.5 cm long) and formalin-inactivated *C. parvum*. In their study, recovery of *C. parvum* was 4 and 6 times greater than the 2.9 and 4.9 µm unmodified CPM. The total removal of the oocysts observed in this study suggests that granular limestone media is more effective for filtration removal of *C. parvum*, when compared to karst limestone material. This is to be expected as, unlike karst limestone media, granular limestone media contain fewer preferential flow paths and are more uniform in conducting particle transport, which most likely resulted in sieving of the oocysts.

Table 3.2: Comparison of collision coefficients with other studies

Comparison of collision coefficients of *C. parvum* derived for various porous media with comparable experimental conditions

Description	This study	Pang et al. (2012)	Abudalo et al. (2010)	Park et al. (2012)	Hijnen et al. (2005)	Harter et al. (2000)
C. parvum	UV-inactivated	Non-inactivated	Formalin-inactivated	Non-inactivated	Non-inactivated	Non-inactivated
Porous media	Limestone sand	Alluvial sand	Ferric oxyhydroxide-coated quartz sand	Ottawa sand	Fluvial gravel (NOM, Fe-ox content)	Alluvial sand
Darcy flow rate (m d^{-1})	1.3	~ 1	0.76	0.71	0.9	0.7
Mean grain size (mm)	0.55	0.78	0.92	0.53	0.5	0.42
Column length (cm)	10	22	10	20	50	10
Solution	10 mM NaCl pH=8	1 mM NaCl pH=7	0.1 mM NaCl pH=5.6–5.8	3 mM NaCl pH=7	River Meuse water EC=569–574 µS cm^{-1} pH=8.0–8.4	degassed tap water EC=220 µS cm^{-1} pH=7.9
Collision coefficient	> 3.93[a]	0.05–0.06	0.26	0.36	0.40[b]	0.56[c]

[a] theoretical value for breakthrough below sample limit of detection (3 oocysts ml^{-1}), [b] correction by Hijnen et al. (2006), [c] calculated by Park et al. (2012) using Tufenkji and Elimelech (2004) method. NOM – naturally occurring organic matter, EC – electrical conductivity.

The collision efficiencies for CPM (α = 1.67–5.41) in the granular limestone media (d_{50}=0.55 mm) calculated for this study (Table 3.1) are two orders of magnitude greater than those of corresponding particles (α = 0.03–0.06) derived for alluvial sand (d_{50}=0.78 mm packed in 22 cm long columns) reported in Pang et al. (2012) although the flow rates (~1 m d^{-1}) and surface charge of porous media were similar in the two studies. The high α values calculated in this study reflect the high removal of colloids in granular limestone media. This supports the untested hypothesis that granular limestone media contains pockets of positive charge that could be effective in removing *C. parvum* oocysts. Table 3.2 compares the collision coefficients of *C. parvum* in sand and gravel media in a number of studies, including fluvial gravels with natural organic matter and ferric oxyhydroxide. The comparison suggests that granular limestone media is more effective in filtration removal of oocysts when compared to other porous media, based on a theoretical collision coefficient calculated from the SLOD for this study. Thus it appears to be a good candidate as a pre-treatment step for drinking water supplies and removal of *Cryptosporidium* oocysts by MAR.

3.4 Conclusions

The results have demonstrated that glycoprotein and biotin-coated CPM are superior to unmodified CPM as surrogates for studying filtration removal of *C. parvum* in granular limestone media, with biotin-coated CPM being slightly more conservative. This finding is consistent with that from the study of alluvial sand by Pang et al. (2012). The lack of breakthrough of *C. parvum* observed in this study suggests that granular limestone media can be used in sand filtration for water treatment and a granular limestone aquifer is suitable for water recycling through MAR.

This study further establishes glycoprotein or biotin-coated CPM as promising surrogates for studying filtration removal of *C. parvum* in porous media. They are easy to work with and can be rapidly detected by using automated counting techniques (e.g. flow cytometry, spectrofluorimetry, and the highly sensitive solid-phase cytometry as used in this study). The low cost of producing the modified CPM is another advantage (Shapiro et al., 2009), making these surrogates cost-effective. Further validation in other porous media and pilot trials for water treatment would allow a more extensive investigation of the usefulness of these newly developed surrogates.

4 Attachment and Detachment Behaviour of Human Adenovirus and Surrogates in Fine Granular Limestone Aquifer Material

Abstract

The transport of human adenovirus, nanoparticles, PRD1 and MS2 bacteriophages was tested in fine granular limestone aquifer material taken from a borehole at a managed aquifer recharge site in Adelaide, Southern Australia. Comparison of transport and removal of virus surrogates to the pathogenic virus is necessary to understand the differences between the virus and surrogate. Since experiments using pathogenic viruses cannot be done in the field, laboratory tests using flow through soil columns were used. Results show that PRD1 is the most appropriate surrogate for adenovirus, when compared to MS2 and 100 nm particles, in an aquifer dominated by calcite material; however, not under high ionic strength or high pH conditions. It was also found that straining due to size and the charge of the colloid were not dominant removal mechanisms in this system. Implications of this study indicate that a certain surrogate may not represent a specific pathogen solely based on similar size, morphology and/or surface charge. Moreover, if a particular surrogate is representative of a pathogen in one aquifer system it may not be the most appropriate surrogate in another porous media system. This was apparent in the inferior performance of MS2 as a surrogate, which is commonly used in virus transport studies.

4.1 Introduction

Artificial stormwater recharge, or managed aquifer recharge (MAR), has become a popular potential drinking water resource in many countries due to its successful attenuation of pathogens, and other substances, in the subsurface and the subsequent possible decrease of drinking water treatment costs. Yet most MAR projects are still in the feasibility stage, usually involving a Quantitative Microbial Risk Assessment (QMRA), and quantitative experiments demonstrating the efficacy of removal are required. One such project is the Salisbury stormwater recharge project near Adelaide, Australia.

A quantitative study of virus transport and removal in aquifer material in the laboratory is therefore a useful first step to investigating field studies. This study compares the transport of human adenovirus (HAdV), a common virus that causes childhood diarrhea, in small columns of fine granular limestone to three different virus surrogates: PRD1 and MS2 bacteriophages and 100 nm carboxylated polystyrene nanoparticles. This was done to assess the suitability of the surrogates for further investigations, possibly in the field, and to

establish preliminary attachment and detachment rates for the colloids in the aquifer material, as well as defining a preliminary removal rate.

HAdV from human fecal sources is present in surface water and groundwater but its transport in groundwater has not yet been widely studied. Prevalent in urban stormwater runoff (Sidhu et al., 2012), HAdV was also detected in a large river and its watershed (Corsi et al., 2014) and has been found in groundwater (Futch et al., 2010), possibly leaking into the subsurface from sewage pipes (Bradbury et al., 2013). Because of its resistance to treatment such as chlorination and UV disinfection (Calgua et al., 2014, Rodriguez et al., 2008), and persistence in sewage and treated water with a very long survival time, HAdV is a conservative pathogenic microorganism. A study in Barcelona, Spain tested samples of effluent from a drinking water treatment plant next to the Llobregat River and found a two log-removal of HAdV, compared to the raw influent water; however, low concentrations of HAdV were still detected, whereas faecal bacterial indicators were no longer detected (Albinana-Gimenez et al., 2006). Due to the persistence of HAdV, it can be used as an appropriate faecal indicator (Ahmed et al., 2010, Bofill-Mas et al., 2006). Although HAdV is present in the environment and demonstrates a higher resistance to environmental factors, few groundwater column test studies have been done using HAdV.

PRD1 has been mentioned in groundwater studies as being a potential surrogate for rotavirus and HAdV (Harvey and Ryan, 2004, Sadeghi et al., 2013, Sinton et al., 1997). Mesquita et al. (2010) suggested that PRD1 may be the best available surrogate for pathogenic HAdV because of its size, structural similarity to HAdV and its long survival time in the environment. PRD1 has been found to be very persistent in soil at ambient temperature (Blanc and Nasser, 1996) and could be a suitable surrogate for HAdV, which also has a very low inactivation rate in the subsurface environment (Sidhu et al., 2010). PRD1 has even been shown to be more conservative than HAdV, exhibiting lower inactivation rates in soil (Davies et al., 2006). Harvey and Ryan (2004), in a mini-review paper about the application of PRD1 in colloidal transport studies, listed the many groundwater transport tests in the field as well as in the laboratory that use PRD1. Transport studies in a limestone aquifer in the Florida Keys, USA, used PRD1 as a virus surrogate and found surprisingly fast travel times in the highly porous limestone bedrock, emphasizing the vulnerability of limestone aquifers to viral contamination (Paul et al., 1995, Paul et al., 1997).

MS2 is widely used as a surrogate for virus transport in groundwater because it is considered a conservative surrogate (i.e. it often over-predicts breakthrough (Schijven et al., 2003), adding a factor of safety) and it is of similar size to many enteroviruses. Transport of MS2 has also been compared to that of nanoparticles. MS2 and PR772 phage were compared to 20 and 200 nm carboxylated latex nanoparticles in fractured dolomite laboratory-scale tests (Mondal and Sleep, 2013). The authors found that the retention of MS2 was lower than retention of similarly sized 20 nm nanoparticles.

Carboxylated polystyrene nanoparticles are commonly used as surrogates to mimic transport of pathogenic microorganisms in groundwater experiments in the field (Bales et al., 1997) and in the laboratory (Tufenkji et al., 2004). Pang et al. (2009) developed protein-coated 20 nm polystyrene nanoparticles to better mimic the transport of viruses in groundwater due to the similar surface charge of the protein-coated particles compared to the viruses.

Column tests in the laboratory using active viruses are not common because working with pathogenic microorganisms is more complicated and requires work in a biological safety laboratory. For this reason, it is not as common to perform column tests with the real pathogen in question. Several studies have done column tests to investigate the transport of viruses in groundwater and to compare to the transport of corresponding surrogates. Hepatitis A, poliovirus, echovirus, coxsackievirus, and Norwalk virus have all been compared to MS2 in column tests. Sobsey et al. (1995) compared the retention of MS2 to that of hepatitis A, poliovirus 1 and echovirus 1 in small (10 cm) soil columns. In 2003, Schijven et al. (2003) published the first paper showing the complete breakthrough curve of pathogenic viruses, (coxsackievirus B4, poliovirus 1) and compared the transport of these viruses to MS2. Even though poliovirus and MS2 are similar in size, there was a difference of 4-log removal in the column tests; MS2 being more conservative and breaking through with very little or no removal. In contrast to poliovirus, coxsackievirus, which is also close in size to MS2, had similar removal compared to MS2. Redman et al. (1997) compared recombinant Norwalk virus (non-infectious) to MS2 and concluded that MS2 is not a good surrogate for Norwalk virus because their electrostatic properties differ, although they are of similar size and shape. Very little attention has been given to HAdV in flow through column experiments until recently (Kokkinos et al., 2015, Pang et al., 2014, Wong et al., 2014) and, to date, there

have not been any column tests in material taken from a deep aquifer, or detachment tests under variable chemical conditions, using HAdV.

The intention of this study is to determine the best surrogate for HAdV and three different possible surrogates were considered: PRD1, MS2 and 100 nm carboxylated polystyrene particles. The surrogates were chosen to test the influence of size, morphology, and surface charge on groundwater transport. HAdV Type 41 has an icosahedral structure and a size of 68 nm (Pang et al., 2014) and is similar in structure and size to PRD1, which is icosahedral and 62 nm in diameter (Ryan et al., 1999). MS2, which is 26 nm in diameter (Pang et al., 2009), is also icosahedral and its surface charge is potentially the most similar to that of HAdV. HAdV is similar in size, although slightly smaller, to the spherical 100 nm nanoparticles, but the icosahedral structure is not shared, nor is the surface charge thought to be similar. By testing these three surrogates in particular, the intention was to determine which mechanism is important for the removal of HAdV, for example, straining or electrostatic interactions. The other goal of this work was to observe the detachment of HAdV under high IS and high pH conditions and to see if a surrogate, such as PRD1, would also detach in a similar manner.

4.2 Materials and Methods

4.2.1 Porous Medium

The aquifer material was collected from a managed aquifer recharge (MAR) site in Parafield Gardens, Adelaide, Australia (for more detail refer to Stevenson et al. (2015)). The tertiary limestone aquifer consists of fine granular limestone with some consolidated chunks. Aquifer material was obtained from core samples taken at a depth of between 170 and 180 m below ground surface. The core material was rinsed in deionized water (DI) and baked in an oven overnight at 80°C before sieve analysis. The material used in the soil column has a grain size of 150 – 250 µm with a D_{50} of 200 µm.

Standard gravimetric methods were used to determine effective porosity. Calculated from the amount of water and material packed in each column, the effective porosity was 0.46 (±0.001) and the bulk density was 1.31 g cm-3 (±0.003). The aquifer material consisted mainly of calcite (63%) and quartz (31.3%) with other minerals including microcline (1.6%),

aragonite (1.5%), albite (0.7%), goethite (0.6%), pyrite (0.5%), ankerite (0.4%), and hematite (0.3%). Geochemical analysis of the material was done by x-ray diffraction analysis.

4.2.2 Column Experiments

The columns were wet-packed in 0.5 cm lifts, while being gently tapped along the side, and were packed with fresh material for each replicate test. 10 cm long glass chromatography columns were used with an internal diameter of 1.5 cm. A flow rate of 1.26 ±0.19 m d^{-1}, mimicking the average flow rate in the aquifer, was injected at the top of the saturated column using a peristaltic pump (Masterflex, Vernon Hills, IL) and the effluent was collected in test tubes at the outflow point at the bottom of the column. At least 50 pore volumes were pumped through the column before tests were begun to equilibrate the chemical conditions of the column. This was done so that the average pH between the influent and effluent solutions did not go above 8.3. The pH of the influent and effluent differed from the average by ±0.5. This is due to the constant leaching of carbonate during the column tests as a result of the high amount of carbonate minerals (calcite and aragonite) present in the aquifer material. The considerable variation of effluent pH observed (between 8.0 and 8.8 for the seven column tests; see Table 4.1) reflects the intrinsic variability of the amount of calcite and aragonite present in the material used for each column test.

Table 4.1: Summary of experimental conditions

Summary of measured experimental conditions: peak concentration (C_{max}), injection concentration (C_0), pulse duration (T_0), influent pH (pH_{in}), effluent pH (pH_{out}).

Colloid	Run	Measured Flowrate (m d^{-1})	C_{max} (pfu ml^{-1} or n ml^{-1})	C_0 (pfu ml^{-1} or n ml^{-1})	C_{max}/C_0	$\log_{10}(C_{max}/C_0)$	T_0 (min)	pH_{in}	pH_{out}
HAdV[a]	1[*]	1.2	1.61E+04	3.29E+07	4.89E-04	-3.31	175	8.0	8.0
HAdV[a]	2	1.2	1.45E+04	4.59E+07	3.15E-04	-3.50	220	7.9	8.7
HAdV[a]	3	1.2	2.26E+03	3.36E+07	6.73E-05	-4.17	210	7.8	8.8
PRD1[a]	2	1.2	6.10E+03	2.40E+08	2.54E-05	-4.59	220	7.9	8.7
PRD1[a]	3	1.2	1.69E+05	2.77E+09	6.10E-05	-4.21	210	7.8	8.8
MS2[b]	4	1.4	2.05E+03	2.15E+07	9.51E-05	-4.02	250	8.1	8.1
MS2[b]	5	1.8	5.80E+01	1.44E+07	4.03E-06	-5.39	200	8.0	8.1
100 nm[c]	6	1.2	2.26E+02	3.33E+07	6.79E-06	-5.17	240	7.9	8.0
100 nm[c]	7	1.0	2.00E+01	1.25E+09	1.60E-08	-7.80	288	8.2	8.5

[*] included 0.5 mM Ca^{2+}, [a] counted by qPCR, [b] counted by plaque assay, [c] counted by epifluorescence microscope

The influent solution consisted of a 10 mM NaCl solution, buffered to a pH of 8.0 (±0.2) with $NaHCO_3$. A small amount of calcium (0.5 mM Ca^{2+} as $CaCl_2$) was used in Run 1 with HAdV, with an overall solution ionic strength (IS) of 10 mM, in order to see if there was any effect

from the calcium. The water temperature was kept at room temperature (between 22 and 23 °C) because the groundwater in the aquifer is 23°C at a depth of 160 to 180 m (Dillon et al., 2008).

Conservative tracer tests were done with 1 mM sodium nitrate ($NaNO_3$) and the effluent concentration of NO_3^- was measured by ultraviolet light absorption of nitrate ions by means of a UV-Vis spectrophotometer (Cary Series UV-Vis, Agilent Technologies, Santa Clara, CA) at a wavelength of 210 nm. Nitrate tests were performed before HAdV and PRD1 tests (but in the same packed columns) because the UV-Vis spectrophotometer was outside of the quarantine area for pathogenic microorganisms and samples to be analyzed could not contain any pathogenic viruses. 40 ml (approximately 4.8 pore volumes) of the tracer solution was injected for each test.

For the detachment portion of the experiment, first 10 ml of DI water (buffered to a pH of 7.0) was pumped through the column (approximately 1.2 pore volumes), followed by 10 ml of a solution high in pH and IS, followed again by 10 ml of DI water. The high pH / high IS solution used was 50 mM glycine with 1.5% beef extract and a pH of 10.

4.2.3 Colloidal Particles

HAdV strain 41 (ATCC VR-930) was cultured in LLK cell line by the Pathology Centre, WA. The virus was then harvested and frozen at -80°C until further use. PRD1 (ATCC_19585-B2; somatic, double-stranded DNA virus, Tectiviridae) was cultured on an *Escherichia coli* (ATCC 11775) host. MS2 (NCTC 12487; F-specific, single-stranded RNA virus, Leviviridae) and host bacterium *Escherichia coli* K-12 Hfr (NCTC 12486) were obtained from the National Collection of Type Cultures (London, UK). The propagation was performed according to ISO 10705-1 (ISO, 1995). Before using the phage stock suspension for the column experiments, it was filtered through a 0.2 µm membrane (Millex-GV, Millipore).

Yellow-green fluorescent carboxylated polystyrene nanoparticles (Fluoresbrite™), with a diameter of 100 nm, were purchased from Polysciences Inc. (Warrington, PA). These nanoparticles/microspheres are commonly used for microbial transport studies in groundwater (Knappett et al., 2008) due to their similarity in size and shape to pathogenic microorganisms and their strong fluorescent intensity.

4.2.4 Zeta Potential

The zeta potential of the porous media was measured using a SurPASS electrokinetic analyzer (Anton Paar, Graz, Austria) based on a streaming potential and streaming current measurement (Luong and Sprik, 2013). The zeta potential measured by the analyzer is related to the surface charge at a solid/liquid interface.

Colloidal particles were tested with an electrophoretic light scattering device (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) and all measurements were performed at least in triplicate. The zeta potential measurements for both the colloidal particles and the aquifer media were done in a solution of 10 mM NaCl, buffered to a pH of 8.0 (±0.2).

4.2.5 Enumeration of Colloidal Particles

Quantitative polymerase chain reaction (qPCR) was used to enumerate the number of HAdV and PRD1 present in the influent and effluent samples.

PCR primers and standards

HAdV were amplified using a previously published primer set (Heim et al., 2003). The PRD1 primer set was designed in this study targeting coat protein gene (GenBank number NC_001421.2) using Primer3 software. A homology search was performed against the GenBank database sequence similarity using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The analysis indicated that the designed primer pair was specific for the PRD1.

The PCR amplified products (i.e. cDNA/DNA) were purified using the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands), and cloned into the pGEM®-T Easy Vector System (Promega, Madison, WI), transferred into *E. coli* JM109 competent cells and plated on LB agar ampicillin, IPTG (isopropyl-β-D-thio-galactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as recommended by the manufacturer. Plasmid was purified using plasmid mini kit (Qiagen).

Purified plasmid DNA containing the PRD1 and HAdV inserts were quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Plasmid copies were calculated, and a ten-fold serial dilution was prepared in DNase and RNase free water to a final concentration ranging from 10^0 to 10^6 copies μl^{-1} and aliquots

were stored at -80°C until use. A 3 µl template from of each dilution was used to prepare standard curves for qPCR.

PCR amplification

Quantitative PCR reactions were performed on Bio-Rad iQ5 (Bio-Rad Laboratories, Hercules, CA), using iQ Supermix (Bio-Rad). Each 25µL PCR reaction mixture contained 12.5 µl of SuperMix, 120 nM of each primer, and 3 µl of template DNA. Bovine serum albumin was added to each reaction mixture to a final concentration of 0.2 µg μl^{-1} to relieve PCR inhibition (Kreader, 1996). For each PCR run, corresponding positive (i.e. target DNA) and negative (sterile water) controls were included. Thermal cycling conditions for PRD1 were: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s, 72°C for 10 s with a final extension of 5 min at 72°C. Thermal cycling conditions for HAdV were as outlined in Sidhu et al. (2010). A melt curve analysis was performed after the PCR run to differentiate between actual products and primer dimmers, and to eliminate the possibility of false-positive results. The melt curve was generated using 80 cycles of 10 s each starting at 55°C and increasing in 0.5°C intervals to a final temperature of 95°C. The melting temperature for each amplicon was determined using the iQ5 software (Bio-Rad).

PCR reproducibility and limit of detection

The reproducibility of the qPCR was assessed by determining intra-assay repeatability and inter-assay reproducibility. The Coefficient of Variation (CV) was calculated using six dilutions (10^6 to 10^0 gene copies) of the PRD1 and HAdV plasmid DNA. Each dilution was tested in triplicate. The CV for evaluation of intra-assay repeatability was calculated based on the threshold cycle (C_T) value by testing the six dilutions six times in the same experiment. The CV for inter-assay reproducibility was calculated based on the C_T value of six dilutions on six different days. To determine the qPCR limit of the detection, known gene copies (i.e. 10^6 to 10^0) of the PRD1 and HAdV were tested with qPCR. The lowest numbers of gene copies that were detected consistently in replicate assays was considered as the qPCR limit of detection.

Enumeration of MS2

The enumeration of infectious MS2 was performed by means of double layer plaque assay according to ISO 10705-1 (ISO, 1995). Briefly, working cultures of the host bacterium were inoculated in Tryptone-yeast extract-glucose broth and incubated at 36 ±2 °C in a water bath while shaking. When the optical density reached a cell-density of approximately 10^8 cfu ml^{-1}

(based on the data obtained in prior calibration measurements), the inoculum culture was taken from the incubator, immediately placed in melting ice to avoid the loss of F-pili by the cells, and used within two hours. Semi-solid Tryptone-yeast extract-glucose agar (ssTYGA) bottles were prepared in advance and the melted agar was kept in a water bath at 45 ±1 °C. A solution of calcium chloride glucose, prepared with 3 ±0.1 g of calcium chloride di-hydrate ($CaCl_2 \cdot 2H_2O$) and 10 ±0.1 g of glucose per 100 ml of DI water, was aseptically added. Supplemented ssTYGA 2.5 ml aliquots were dispensed in capped culture tubes placed in a water bath until used. To each tube, 1 ml of the sample, or dilutions thereof, and 1 ml of the inoculum culture were added. Finally, tubes were carefully mixed and poured over the surface of Tryptone-yeast extract-glucose agar plates, distributed and allowed to solidify. Plates were incubated upside-down at 36 ±2 °C for 18 ±2 hours and the plaques were counted. Positive and negative controls were performed for each test series.

Even though qPCR was used to enumerate HAdV and PRD1, which does not indicate only infectious viruses but rather enumerates all gene copies present dead or alive, it can be assumed that for the short time period of the experiments, inactivation was minimal. As mentioned previously, HAdV and PRD1 can survive for long periods in the subsurface. For the column tests using MS2, inactivation batch tests were performed to monitor the MS2 suspension, which remained stable throughout the duration of the experiments; however, inactivation may have been caused when the MS2 came into contact with the porous media, resulting in an underestimation of MS2.

Enumeration of 100 nm particles

The 100 nm nanoparticles were enumerated using a Nikon Eclipse 8000 epifluorescence microscope with a 100 × magnification objective (final magnification: 1000 ×). The microscope is equipped with an automatic stage that can be driven by the user such that the whole filtration area could be scanned. 100 µl of each sample was pipetted onto a filter area 4 mm in diameter, delineated with a fine black permanent marker, on a white Millipore Isopore™ membrane filter (Billerica, MA) with a pore size of 0.05 µm. The filter was then placed on a metal holder (AES Chemunex, bioMérieux, Marcy l'Étoile, France) with 10 µl of phosphate buffer solution to hold the filter in place. Scanning the whole 4 mm diameter area delineated in black allowed a detection limit of one nanoparticle per 100 µl sample; however, the limit of quantification is higher due to random sampling error (intrinsic variability) and operational variability (4 particles per filter if a relative precision of 50% is

accepted (Stevenson et al., 2014)). This method of scanning the whole filter has been used by Knappett et al. (2008) to enumerate 1.5 µm carboxylated polystyrene particles and Bales et al. (1997) have used an epifluorescence microscope to count 100 nm carboxylated polystyrene particles (although not on the whole filtration area).

4.2.6 Data Analysis

Filtration efficiency

The removal of the particles was quantified using colloid filtration theory (CFT). CFT calculates the collision efficiency, α [-], and can be determined from the following formulas:

(Pieper et al., 1997)

$$\alpha = \frac{d\left[\left(1 - 2\frac{\lambda}{x}\ln(RB)\right)^2 - 1\right]}{6(1-\theta)\eta\lambda} \quad (1)$$

(Tufenkji and Elimelech, 2004)

$$\alpha = -\frac{2}{3}\frac{d}{(1-\theta)\eta x}\ln\left(C/C_0\right) \quad (2)$$

$$\alpha = \frac{2}{3}\frac{d\theta}{(1-\theta)\eta U}k_{a1} \quad (3)$$

where d is the mean grain size [L], λ is dispersivity [L], x is the length of the column [L], RB is relative mass recovery [-], θ is effective porosity [-], η is the single-collector efficiency [-], C/C_0 is the normalized concentration of the tracer at breakthrough plateau [M M^{-1}], k_{a1} is an attachment rate coefficient [T^{-1}], explained in more detail in the next section, and U is Darcy velocity [L T^{-1}]. RB was estimated by integrating the area under the breakthrough curve. Darcy velocity (U) and dispersivity (λ) are needed for the calculations of η and α. The measured flow rates were used for the U values, while dispersivity (λ) was found by simulating the colloid breakthrough curves in HYDRUS-1D (Šimůnek et al., 2013). The η and α values were calculated based on the equations of (Tufenkji and Elimelech, 2004). A Hamaker constant of 7×10^{-21} J for PRD1 (Ryan et al., 1999) was used for PRD1 and HAdV. A buoyant density of 1.085 g cm^{-3} was used by Hijnen et al. (2005) to represent MS2 and *E. coli*, which covers the size range of the microorganisms.

Eq. 1 is useful for colloid transport in field studies whereas Eq. 2 is more appropriate for column studies. Both equations were used for comparison because Eq.1 has the advantage of calculating α with the relative mass recovery (*RB*), which includes the attachment and detachment portion of the breakthrough curves. The observed data was applied to Eq. 1 to calculate α since *RB* could be determined; however, for Eq.2, the maximum value from the HYDRUS-1D modelled values was used for C/C_0 due to the fact that the observed values did not produce a steady C_{max}. Eq. 3 was also employed for comparison, since it is calculated using the first attachment rate coefficient, k_{a1}, and is not dependent on steady breakthrough.

Transport modelling

The software package HYDRUS-1D (Simunek et al., 2013) was used to model the breakthrough curves of the conservative tracer, nitrate, and two of the colloids tested: HAdV and PRD1. Advection-dispersion equations for colloid transport are implemented in a numerical model using a two-site attachment/detachment model and the following formulas (Schijven & Šimůnek, 2002):

$$\frac{\partial C}{\partial t} + \frac{\rho_b}{\theta}\frac{\partial S_1}{\partial t} + \frac{\rho_b}{\theta}\frac{\partial S_2}{\partial t} = \lambda v \frac{\partial^2 C}{\partial x^2} - v\frac{\partial C}{\partial x}$$

$$\frac{\rho_b}{\theta}\frac{\partial S_1}{\partial t} = k_{a1}C - \frac{\rho_b}{\theta}k_{d1}S_1 \qquad (4)$$

$$\frac{\rho_b}{\theta}\frac{\partial S_2}{\partial t} = k_{a2}C - \frac{\rho_b}{\theta}k_{d2}S_2$$

where C is the concentration of the tracer [M L^{-3}], t is time [T], ρ_b [M L^{-3}] is dry bulk density, θ [-] is effective porosity, S_1 and S_2 are the concentrations of sorbed particles at the first and second kinetic sorption sites [M M^{-1}], λ is dispersivity [L], v is pore water velocity [L T^{-1}], x is distance along the flow path [L], k_{a1} and k_{a2} are attachment rate coefficients [T^{-1}] and k_{d1} and k_{d2} are detachment rate coefficients [T^{-1}] at the first and second kinetic sorption sites. To determine the bias, the observed breakthrough curve was compared to the modelled curve by finding the log-difference of the averages as well as of the 95th percentiles. Inactivation was not modelled because, for the short duration of the experiments, inactivation was not an issue for HAdV and PRD1.

4.3 Results and Discussion

4.3.1 Porous Media and Colloid Characterization

Zeta potential (ζ) measurements were done for all colloids in 10 mM NaCl, pH 8.0 (±0.2) and was found to be -15.0 mV (±2.0) for the HAdV used in this study. Wong et al. (2012) measured a ζ value of approximately (read from graph) -24 mV for HAdV serotype 2 in 10 mM NaCl at pH 8. Pang et al. (2014) measured ζ values of -24 mV and -27 mV for purified HAdV type 41 and MS2, respectively, in 2 mM NaCl, pH 7. The MS2 used in this study had a ζ value of -16.6 mV (±1.3), which was most like the ζ value of HAdV, and PRD1 had a ζ value of -7.8 mV (±2.6). The ζ value of PRD1 was measured to be -9.8 mV by Mesquita et al. (2010).

In this study the 100 nm carboxylated polystyrene nanoparticles had a ζ value of -70.4 mV (±2.8) compared to approximately -42 mV (read from graph) that was measured by Pang et al. (2009) for 20 nm carboxylated polystyrene particles (Bangs Laboratories, Inc. [Fishers, IN]) in 1 mM NaCl, pH 8 and -37 mV for 70 nm carboxylated silica particles (Micromod Partikeltechnologie GmbH [Germany]) in 2 mM NaCl, pH 7 (Pang et al., 2014). The studies chosen for comparison differ by similarity in particle material (Pang et al., 2009) and size (Pang et al., 2014) with this study, but measurements of ζ can vary depending on the ionic strength and pH, as well as measuring instrument used and the colloid manufacturer. In the two studies mentioned for comparison, the measuring instrument was the same as that used in this study, though the colloid manufacturers differed and the solution chemistries. The measurements of ζ in this study were generally less negative (except for the 100 nm particles) than the measurements found in comparable studies; however, the measurements relative to each other (i.e. nanoparticles were most negative, PRD1 least negative) were consistent with other studies. The ζ value of the porous media used in this study was -38.7 mV (±0.9).

4.3.2 Column Tests

Table 4.1 lists the test conditions and measured parameters of each of the seven column test runs. Log-removal ($\log_{10}$ of C_{max}/C_0), which is not dependent on detachment because it is only looking at the initial attachment phase, was highest for the 100 nm particles and MS2. The log-removal of PRD1 was slightly higher than that of HAdV. Run 1 of HAdV had the least log-removal, even though 0.5 mM Ca^{2+} was added to the solution which usually causes more colloidal removal (Bales et al., 1991, Sadeghi et al., 2013). The system in this study is

dominated by calcite and it may be that adding a small amount of Ca^{2+} produced a negligible effect.

No clumping of the 100 nm particles was observed under the microscope, but the number of nanoparticles that did break through was below the limit of quantification. The 100 nm particles, being the largest of the colloids tested, may have been subject to straining. Although, this seems unlikely, due to the small ratio of colloid diameter to mean grain size (< 0.0017) (Bradford et al., 2002). An exception has been hypothesized by Tufenkji et al. (2004) that angular porous media, such as the material used in this study, can result in irregular packing which in turn can cause much smaller pore spaces than those predicted using the mean grain size. Nevertheless, if straining were the only removal mechanism, then the much smaller MS2 would have broken through significantly more than the 100 nm particles, which was not the case in this study. This is difficult to confirm without quantifying the surface inactivation rate of MS2 that has come into contact with the porous media.

4.3.3 Filtration Removal of Colloids

Colloid filtration theory was used to compare the removal of HAdV to PRD1. The contact efficiency (ratio of rate at which colloids collide with porous media to the rate at which colloids flow), η, was 0.19 for both PRD1 tests and 0.14 for all three HAdV tests (Table 4.2). This means that, in theory, there are more collisions of PRD1 with the granular limestone. The collision efficiency (the number of successful contacts that result in attachment divided by the number of collisions) was calculated using three different equations: for field tests (α_1), for column tests (α_2) and using the first attachment rate coefficient (α_3). The results (Table 4.2) show that α_1 was lower for the PRD1 tests, compared to the HAdV tests, demonstrating less removal by attachment to the porous media or more detachment of PRD1. Since α_1 is dependent on the relative mass recovery (*RB*), it also reflects the higher detachment of PRD1 under high pH conditions, relative to HAdV. Additionally, Run 3 was run twice as long as Run 2 and should have more detachment; this is reflected in the relatively lower α_1 values for Run 3. Run 1, which was only run for the attachment portion of the experiment, had the highest α_1 value.

Table 4.2: Calculated collision coefficients

Comparison of collision coefficients: observed relative mass recovery including detachment (RB), single collector factor (η), collision coefficient for field including detachment (α_1), collision coefficient for column tests (α_2), collision coefficient calculated from k_{a1} (α_3), average of three collision coefficients (α_{ave}), standard deviation of collision coefficients (SD).

Tracer	Run	RB	η	α_1	α_2	α_3	α_{ave}	SD
HAdV	1	2.63E-04	0.14	0.154	0.133	0.008	0.099	0.079
HAdV	2	7.22E-04*	0.14	0.138*	0.165	0.180	0.161	0.021
HAdV	3	1.49E-02*	0.14	0.078*	0.177	0.054	0.103	0.065
PRD1	2	8.79E-03*	0.19	0.063*	0.136	0.154	0.118	0.048
PRD1	3	7.40E-01*	0.19	0.004*	0.125	0.038	0.056	0.062

* Includes detachment

The values of α_2 and α_3 are higher than the α_1 values (with the exception of Run 1 [no detachment] and α_3 for HAdV, Run 3) because detachment is not taken into account, hence the higher removal. Similar to the α_1 values, the α_2 and α_3 values are higher for HAdV, when compared to PRD1. This further supports the claim that there is less removal of PRD1. In contrast, it appears that there is less removal of HAdV in Figure 4.1 (Graph B), by one order of magnitude. This ambiguity infers that PRD1 may not be a perfect surrogate for HAdV, but the best of the surrogates tested. Even though detachment was not taken in account, the values of α_2 and α_3 are lower for Run 3. Thus, it appears that not only the detachment caused less removal for Run 3, but there was also less attachment. The averages and standard deviations of the three collision coefficients are shown in Table 4.2 and demonstrate the high variability of the calculated α-values. The values of η and α were not calculated for MS2 or the 100 nm particles because the breakthrough was almost entirely below the limit of quantification for these colloids.

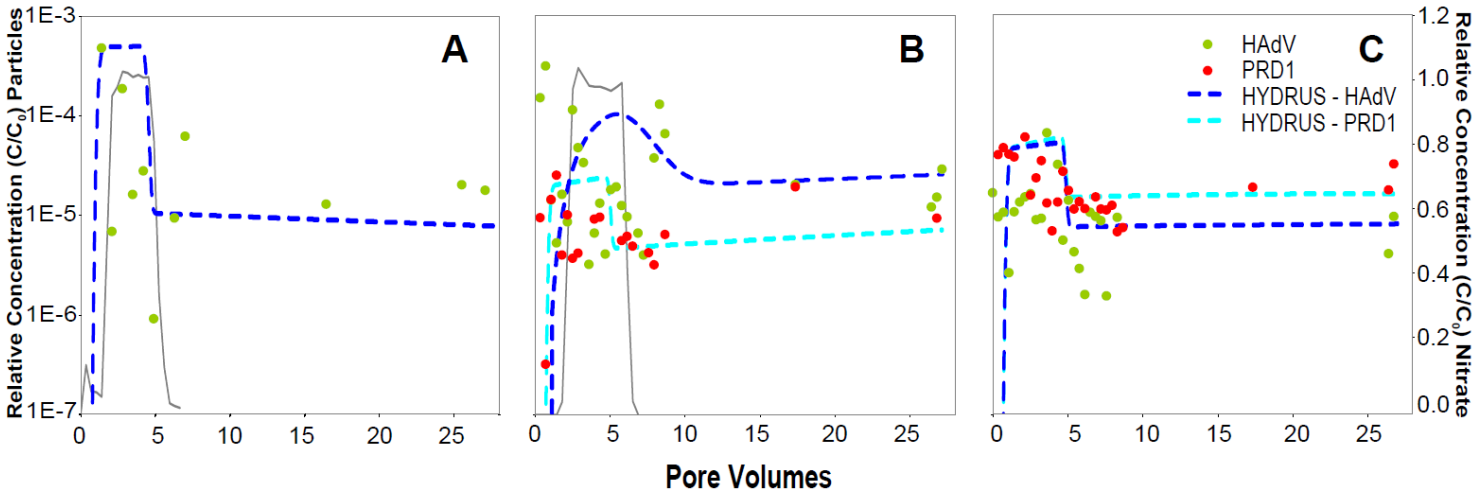

Figure 4.1: Breakthrough curves for human adenovirus and surrogates

Observed breakthrough curves of HAdV and PRD1 for test runs 1 (A), 2 (B), and 3 (C) and simulation results of modelling performed in HYDRUS-1D. Nitrate breakthrough curves are shown in gray for runs 1 (A) and 2 (B), right y-axis.

Table 4.3: Summary of calculated and modelled parameters

Summary of calculated and modelled parameters using HYDRUS-1D: pore water velocity (ν), dispersivity (λ), peak concentration (C_{max}), injection concentration (C_0), attachment rate coefficients at first and second sorption sites (k_{a1}, k_{a2}), detachment rate coefficients at first and second sorption sites (k_{d1}, k_{d2}), and log-differences of observed to simulated data (average and 95th percentile).

Tracer	Run	ν (cm min^{-1})	λ (cm)	C_{max}/C_0	$\log_{10}(C_{max}/C_0)$	k_{a1} (min^{-1})	k_{d1} (min^{-1})	k_{a2} (min^{-1})	k_{d2} (min^{-1})	R^2	Observed		Simulated		log (obs/sim)	
											Average	95%	Average	95%	Ave.	95%
nitrate	1	0.10	0.15	1.00E+00	0	-	-	-	-	0.99	-	-	-	-	-	-
nitrate	2	0.11	0.09	1.00E+00	0	-	-	-	-	0.95	-	-	-	-	-	-
HAdV	1	0.13	0.08	4.98E-04	-3.30	0.006	0.0003	0.1	0	0.26	5.72E-05	2.80E-04	6.37E-05	4.94E-04	-0.05	-0.25
HAdV	2	0.15	0.08	1.02E-04	-3.99	0.15	0.00009	0.12	0.05	0.19	4.00E-05	1.45E-04	3.30E-05	9.59E-05	0.08	0.18
HAdV	3	0.15	0.08	5.24E-05	-4.28	0.045	0.0003	0.12	0	0.16	1.10E-05	2.76E-05	9.81E-06	1.67E-05	0.05	0.22
PRD1	2	0.15	0.08	2.38E-05	-4.62	0.18	0.0001	0.0001	0.0001	0.03	5.21E-06	1.75E-05	7.91E-06	2.24E-05	-0.18	-0.11
PRD1	3	0.15	0.08	5.93E-05	-4.23	0.045	0.0006	0.12	0	0.03	2.11E-05	4.55E-05	1.66E-05	2.05E-05	0.10	0.35

4.3.4 Transport of Colloids

Modelling

To characterize the transport of HAdV and surrogates in the porous media, it was attempted to model the breakthrough curves using HYDRUS-1D. The intention was to model the nitrate breakthrough curves in order to determine the flow rate and dispersivity, however, this turned out to be a challenge. Therefore, different flow rates and dispersivities were used for the conservative tracer and the colloids, as can be seen in Table 4.3.

The pore water velocity (v) and dispersivity (λ) of the conservative tracer and the colloids were calculated separately since the granular limestone is quite diverse in shape and would not result in a uniform packing. In some heterogeneous material, size exclusion of the colloids can play a role and colloidal transport can be controlled by advective processes, with less λ than solute tracers and higher v (Pang et al., 2005). This effect was seen in the material used in this study where the conservative tracer (nitrate) had v values of 0.10 – 0.11 cm min^{-1} and the colloids 0.13 – 0.15 cm min^{-1}. The λ of the nitrate tracer was modelled to be 0.09 – 0.15 cm and all the colloids had a λ of 0.08 cm. Since the nitrate tests were analyzed outside of the quarantine area used for pathogenic microorganisms, it was necessary to run the conservative tracer test ahead of the test using pathogenic viruses. Consequently, it is possible that the tests were run slightly slower or faster, but this was considered negligible since the sample volumes were consistent. The breakthrough of the viruses preceded the breakthrough of the conservative tracer, demonstrating preferential flow due to the heterogeneous nature of the material and packing. This is known as 'velocity enhancement' (Pang et al., 2005), when the preferential flow path acts as a high speed motorway for the colloids while the solute slowly disperses into the smaller pore spaces.

The data from the colloid column experiments was scattered, a challenge when quantifying low concentration samples which can have relatively high intrinsic variability, and almost impossible to model. Only the constant IS portion of the breakthrough data was modelled. Figure 4.1 shows the breakthrough curves for all the HAdV and PRD1 tests. PRD1 appears to attach and detach more than HAdV and the attachment and detachment values are the same or slightly higher for PRD1, except for the secondary attachment/detachment coefficients for Run 2 (Table 4.3). In Figure 4.1 (Run 2, Graph B), it is apparent that there is high, continual (perhaps momentarily increasing) detachment of HAdV and PRD1 because the tails of the breakthrough curves are rising even though viruses cannot multiply on their

own outside of a host cell. The R^2 values for the HAdV tests ranged from 0.16 to 0.26 and the R^2 value for both PRD1 tests was 0.03, which signifies that there was almost no fit at all. The standard errors of the calibrated parameters were high and the solutions were not unique, producing variable results depending on the initial estimates. Consequently, the results of the HYDRUS model cannot be trusted and, as an alternative method of comparison, the log-differences of the averages and 95th percentiles were calculated (displayed in Table 4.3).

There was minimal to no breakthrough for column tests done with MS2 and 100 nm particles; breakthrough was below the limit of quantification. A lower count of MS2 might be expected due to the fact that plaque forming analysis generally enumerates fewer microorganisms than qPCR but, on the contrary, Pang et al. (2014) found that MS2 (enumerated by plaque forming analysis) exhibited higher breakthrough in saturated sand columns when compared to HAdV (enumerated by qPCR). Even so, it has been shown that media containing iron oxide (e.g. pyrite, hematite and goethite) can cause surface inactivation due to the strong electrostatic interaction with negatively charged viruses and is more notable in the inactivation of MS2 relative to PRD1 (Ryan et al., 2002). The breakthrough of the nanoparticles was also quite low, probably because the nanoparticles had a very negative surface charge (~ -70 mV), even though the limit of quantification for enumerating the nanoparticles was very low (4 particles per filter based on Poisson distribution (Stevenson et al., 2014)).

Due to the low breakthrough, it was not attempted to model the breakthrough curves for MS2 or the 100 nm particles. Interestingly, MS2 is usually used as a conservative surrogate, although in this study it was the least conservative, along with the 100 nm particles. The fact that MS2 and the 100 nm particles broke through minimally demonstrates that straining was not a removal mechanism and that the size and structure of PRD1 influences attachment more than surface charge.

4.3.5 Detachment of viruses

Attachment of viruses was very reversible. Column elution tests were done by alternating injection of 1.2 pore volumes of DI water and 50 mM glycine (1.5% beef extract), pH 10. Figure 4.2 shows how the HAdV and PRD1 colloids attach and become a constant source of contamination that can be aggravated by changes in the chemistry of the influent water i.e. high IS and pH. Interestingly, PRD1 detaches much more than HAdV and is less negatively

charged than HAdV, hence not as strongly bonded to patches of positive charge present on the surface of the aquifer material. Relative to PRD1, HAdV detaches minimally (see Figure 4.2) after a cycle of DI water and glycine, which is apparent in the lower relative mass recovery of HAdV. Both tests show that the relative mass recovery of HAdV is one order of magnitude lower than that of PRD1 (Table 4.2). For Run 2 HAdV and PRD1 have a lower relative mass recovery, compared to the other test runs, because this test had a much shorter duration: 54 compared to 109 pore volumes.

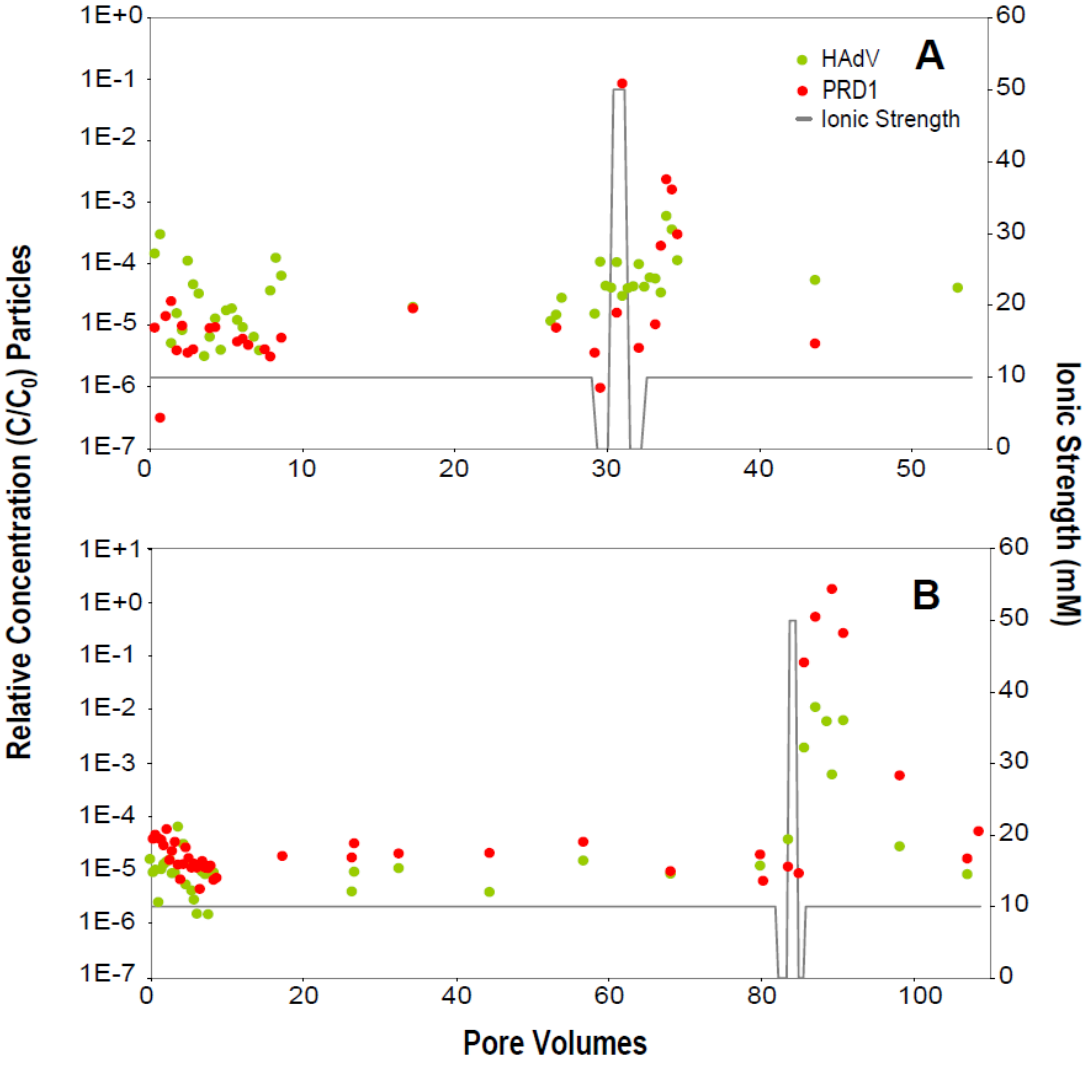

Figure 4.2: Detachment curves for human adenovirus and PRD1 phage

Detachment curves for HAdV and PRD1 for test runs 2 (A) and 3 (B). Ionic strength (gray curve) is plotted relative to the right y-axis.

During the detachment tests, two peaks occurred (Figure 4.2), at pore volume 31 and 34 for the first run and at 87 and 89 for the second run. The beginning of injection of the two DI

water cycles is approximately 2 pore volumes apart and the two detachment peaks are separated by 2 to 3 pore volumes. The second peak, for both test runs, coincides with a significant amount of brown material observed in the effluent samples. There was no suspended fine material observed in the samples taken during the first peak. The release of aquifer material during the second peak indicates that the high IS water followed by DI water caused the release of viruses with, and perhaps attached to, the aquifer material.

A study by Bradford and Kim (2010) looked at the effect of different cycles of DI water and high IS water on the removal of clay. They found that the highest clay removal occurred during the DI phase and that the most clay was removed when the highest IS was injected just before the DI. The authors used 0.1, 1, 10 and 100 mM NaCl, as well as 100 mM $CaCl_2$. Tosco et al. (2009) also found that colloids were released during the DI water phase of an experiment. The authors injected carboxylated latex microspheres into sand columns with different IS solutions (between 1 and 300 mM NaCl at pH 6.8), followed by a flushing of DI water, to test the effect of IS on the transport and release of colloids. The authors found the highest release of colloids during the DI phase after experiments of high IS (30 to 300 mM) and they observed that no particles were released after an IS of 1 to 10 mM, followed by DI water.

Whereas in the studies by Bradford and Kim (2010) and Tosco et al. (2009), the release happened during the DI water injection, the system in this chapter exhibits a delayed response, which may be due to the different porous media used. The two studies mentioned used columns of sand and in this chapter the material in the column was 63% calcite; the naturally occurring Ca^{2+} adds complexity to chemical changes in this system. The detachment experiments in this study show that strongly attached HAdV could be a constant source of contamination in a limestone aquifer and could cause a constant slow release of viruses, and a large release, when aggravated by changes in groundwater chemistry.

4.4 Conclusions

Managed aquifer recharge is becoming a popular initial treatment option for drinking water due to its efficiency and low costs, however, injecting stormwater runoff into the subsurface can introduce pathogenic microorganisms into groundwater. This study considered the

transport of HAdV in the subsurface, and compared the transport and removal to various surrogates that are diverse in size, morphology and surface charge. Of the three surrogates tested PRD1 is the best surrogate for HAdV. MS2 and the 100 nm carboxylated polystyrene particles showed minimal breakthrough and did not mimic the transport and removal of HAdV. We looked at the dominating mechanisms of removal and the possible colloid characteristics that could influence this. With MS2 and the 100 nm particles, the possibility of straining being a removal mechanism was tested. If straining were an issue, then the MS2 would have broken through more than the other three colloids, which it did not. Because MS2 was not transported like HAdV, it can also be concluded that surface charge is not a dominating factor. PRD1 has a similar size, shape and structure to HAdV and was the best surrogate for aquifer material dominated by calcite, but it could not model the magnitude of detachment of HAdV under high IS / high pH conditions. Therefore, size, morphology, and perhaps the surface macromolecules are the dominant characteristics that control the fate and transport of HAdV in fine granular limestone material. This study demonstrates the importance of testing the surrogates and comparing them to the real pathogenic microorganisms in laboratory tests using the aquifer media from the site under consideration. Implications of this study could influence how field tests using bacteriophages and nanoparticles are interpreted.

5 Overall Conclusions and Suggestions for Further Work

The goal of this doctoral work was to establish a method to detect and enumerate low concentrations of colloids in groundwater and to find appropriate surrogates for two different pathogenic microorganisms and to test one under variable chemical conditions. These goals were formulated into three main questions:

- Pathogenic microorganisms, as well as indicators and tracers (when used), are present in groundwater at low concentrations. For example, directly below a septic tank containing infectious coxsackievirus A9, the virus concentration was 0.003 infective units per litre at an on-site sewage disposal system in Florida. Three metres down gradient from this point, the viruses were already undetectable (Anderson, 1991). How can very few target microorganisms be detected and enumerated in what quantity of water? What is the limit of detection and quantification?
- The surface charge and macromolecules present on the surface of a microorganism can affect their attachment and detachment to substrate in the subsurface, and therefore their transport and removal. Considering three different coatings on microspheres, what is the best surrogate (mimics transport well) for *Cryptosporidium parvum* in granular limestone material with high carbonate content?
- Human adenovirus (HAdV) is a persistent virus that is found in groundwater, but has not been studied extensively. What is the best surrogate for HAdV and what is the dominant removal mechanism of HAdV in fine granular limestone material: size, morphology or surface charge? Does this surrogate respond similarly under detachment conditions i.e. high ionic strength and high pH event in groundwater?

These questions were addressed in three chapters. Chapter 2 discussed the limits of detection and quantification of different sizes of microspheres in various background matrices using solid-phase cytometry as the method of enumeration. It was found that fluorescent surrogates could be reliably detected in very turbid water down to a size of 0.75 µm and down to a size of 0.5 µm in groundwater. The maximum sample size that could be

filtered of highly turbid surface water was 1 ml and 500 ml of pristine spring water could be filtered without causing the solid-phase cytometer to abort.

Using the enumeration method developed in Chapter 2, Chapter 3 went on to investigate the removal and transport of 4.5 µm microspheres in granular limestone aquifer material and were compared to two different strains of *C. parvum*, an oocyst that causes gastrointestinal illness. Three different types of microspheres were compared: the standard carboxylated microspheres and biotin- and glycoprotein-coated microspheres. It was found that glycoprotein-coated microspheres were the best surrogate for *C. parvum*, in terms of mimicking the transport and removal, in granular limestone material. This was to be expected, as *C. parvum* naturally contains glycoprotein on its surface.

In Chapter 4, the transport of carboxylated nanoparticles, MS2 and PRD1 bacteriophages was compared to that of HAdV in fine granular limestone material. The comparison of these surrogates shed light on the dominant removal mechanism of this pathogenic microorganism. This chapter showed that size and morphology of HAdV affect removal more than the surface charge, due to the fact that the surrogate with the most similar surface charge (MS2 phage) did not mimic the transport of HAdV. This is a significant finding due to the fact that MS2 phage is often used as a surrogate to represent virus transport in the subsurface, but may not be the ideal conservative surrogate that it is thought to be. The best surrogate, of the three tested, to represent HAdV was found to be PRD1 phage, but this surrogate did not demonstrate a similar detachment pattern when compared to HAdV under high ionic strength and high pH conditions.

An obvious discrepancy between experiments run using microorganisms and those run using microspheres is due to the issue of inactivation. When microspheres or nanoparticles are used, it is necessary to determine the inactivation rate of the targeted microorganism in separate experiments. *C. parvum* and HAdV are often used as indicator microorganisms because of their long survival time and resistance to inactivation. In Chapters 2 and 3 *C. parvum* and HAdV, as well as the surrogate PRD1 phage, were enumerated with methods that counted all the cells, whether inactivated or not. MS2 phage, on the other hand, was enumerated with the plaque forming method, which only counts living cells. This may have resulted in an underestimation of MS2. Another explanation for the high removal of MS2 is the presence of multivalent cations (Ca^{2+}) in porous media with a high carbonate content

and its tendency have higher removal rates in hydrophobic material (Schijven and Hassanizadeh, 2000). Material with high hydrophobicity usually contains a large amount of organic carbon, but this was not measured in this work.

These conclusions help to illuminate the challenges and to offer solutions to the problem of predicting the transport and removal of pathogenic microorganisms in the subsurface. Two strategies were developed to further advance colloid tracer technology and the enumeration of colloids. The implication of this work is that each system comprising of aquifer media, chemical conditions and a specific microorganism, is unique and needs to be considered on a case by case basis. Developing and using the most realistic surrogate possible to imitate the transport and removal of a pathogenic microorganism in the subsurface is, therefore, of utmost importance. Using a randomly chosen surrogate in subsurface experiments may not represent any targeted pathogen and could result in unrealistic conclusions. This work offers an alternative approach to quantify the potential of contamination of a drinking water well rather than the standard policy in Austria, as well as many other countries, of defining a 60-day groundwater travel time. The alternative method suggests determining transport parameters by way of field tests using the proposed surrogates and using these parameters in a groundwater model to predict the appropriate protection area for a drinking water well. Quantitative Microbial Risk Assessment (QMRA) software is being developed (Kroiss, 2006), programmed in MATLAB®, that uses these parameters as input to calculate the safe setback distance of a well from a potential source of contamination. Schijven et al. (2010) have already formulated an empirical model for calculating the safe setback distance for a drinking water well in an unconfined sandy aquifer, which could be incorporated into a QMRA model.

Future work should focus on tracer tests at a field site using glycoprotein-coated microspheres to represent *C. parvum* and PRD1 phage to represent HAdV. Of the surrogates considered, these were the most successful at imitating the transport of these particular pathogens, although other alternatives could be tested in the future. Technology using ultrafiltration, the filtration and concentration of large volumes of sampled water, is currently being developed and will aid with enumerating low concentrations of surrogate breakthrough during tracer tests (Kunze et al., 2015), which is more representative of the pathogens that are present in the environment. As a step prior to doing experiments in the field, the surrogates should be tested further under various diverse conditions. Column tests

in the laboratory could be done using hydrophobic conditions, different amounts of dissolved organic carbon (DOC), as well as conditions of varying ionic content. More specifically, the behaviour of the surrogates could be tested in the presence of multivalent cations (such as Al^{3+}) and in hydrophobic material, for example, using organosilane-coated silica sand (Farkas et al., 2015). As mentioned above, multivalent cations and hydrophobicity can influence the transport of microorganisms in porous media, necessitating further testing under these conditions.

6 Notation

C	concentration of the tracer [M L^{-3}] e.g. mg m^{-3} or pfu m^{-3}
C_0	influent concentration of the tracer [M L^{-3}]
d or d_{50}	mean grain size [L]
d_{60}	grain size when 60% passes sieve [L]
d_{10}	grain size when 10% passes sieve [L]
k_{a1}, k_{a2}	attachment rate coefficients [T^{-1}]
k_{d1}, k_{d2}	detachment rate coefficients [T^{-1}]
RB	relative mass recovery [-]
S_1, S_2	concentration of sorbed particles at kinetic sorption sites [M M^{-1}]
t	time [T]
U	Darcy velocity [L T^{-1}]
x	transport distance [L]
α	collision efficiency [-]
α_1	collision coefficient for field [-]
α_2	collision coefficient for column tests [-]
α_3	collision coefficient calculated from attachment [-]
ζ	zeta potential [M L^2 T^{-3} I^{-1}] e.g. mV
η	single-collector efficiency [-]
ϑ	effective porosity [-]
λ	dispersivity [L]
v	pore water velocity [L T^{-1}]
ρ_b	dry bulk density [M L^{-3}]
ϕ	diameter of the colloid [L]

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Appendix A

Table A1: Solid-phase cytometer settings

Discriminant settings used for the Chem*Scan*™ RDI (AES Chemunex, Ivry sur Seine, France). The settings are an edited version of the Vibrio G2000 2Mhz.APP provided by the manufacturer

Discriminant Settings	Minimum	Maximum
S/P Area Ratio	0	1
T/P Area Ratio	OFF	OFF
Single Line Samples	500	-
Samples	1	250
Lines	1	60
Peak Intensity	250	65535
Peaks Per Line	-	2
Wiggles Per Line	-	6
Half Width	-	15
Specific Intensity (AS)	3	-
Specific Intensity (HW)	10	-
2D Gaussian Fit	-	1000

Table A2: Variation coefficients and p-values from Mann-Whitney U-test for dilution series with fluorescent beads spiked in different waters. Variation coefficients (ratio of standard deviation to the mean) less than approximately 0.50 are considered to indicate precision and corresponds to the lowest number that can be quantified (= quantification limit), i.e. 4 particles (ISO, 2005). Calculated p-values from Mann-Whitney U-test show significant differences of measuring beads with SPC in environmental water samples compared to sterile water; multiple comparisons were done for 0.5 µm beads, dual comparisons for 0.75 and 1 µm beads

Dilution beads filter^{-1}		Variation coefficients				Mann-Whitney p-values			
		10^0	10^1	10^2	10^3	10^0	10^1	10^2	10^3
0.5 µm	Sterile	0.52	0.20	0.08	0.03	-	-	-	-
	AGW1	0.43	0.10	0.21	0.07	> 0.1	> 0.05	> 0.1	> 0.1
	Danube	0.00	0.34	0.27	0.06	> 0.1	> 0.1	> 0.1	> 0.1
	Neusiedler See	0.69	0.21	0.15	0.12	> 0.1	> 0.1	< 0.05	> 0.05
	Oberer Stinkersee	1.15	0.20	0.43	0.19	< 0.05	< 0.05	< 0.001*	< 0.001*
0.75 µm	Sterile	0.20	0.08	0.09	0.04	-	-	-	-
	Oberer Stinkersee	0.47	0.14	0.10	0.03	> 0.1	> 0.1	> 0.1	> 0.1
1 µm	Sterile	0.97	0.03	0.11	0.05	-	-	-	-
	Oberer Stinkersee	0.00	0.30	0.01	0.02	> 0.1	> 0.1	> 0.1	> 0.1

* statistically significant (< 0.05 for dual comparisons, < 0.01 for multiple comparisons according to Bonferroni correction)

Enumeration method of *C. parvum*

The method of dying the oocysts with *EasyStain*™ antibody (BTF Pty. Ltd., bioMérieux, North Ryde, Australia) and enumerating them with solid-phase cytometry (SPC) (Chem*Scan*® RDI, AES Chemunex, bioMérieux, Ivry sur Seine, France) is described here in more detail. The filtration device was cleaned with EtOH 70% and distilled water before filtering 1 ml of each sample onto CB04 Chem*Scan* Filters (bioMérieux). On a petri plate the filters were each placed on 20 µl of *EasyStain* and 30 µl of sterile deionised (DI) water and incubated for 15 minutes at 37°C. After incubation, the filters were allowed to dry on absorbent paper and then placed on 150 µl of fixing buffer (bioMérieux) on a petri plate. The filters were then incubated for 2 minutes at room temperature, after which they were placed again on absorbent paper to dry. The Chem*Scan* holders were cleaned with EtOH 70% before pipetting 100 µl of phosphate buffer solution onto the holder and placing a support pad (bioMérieux) onto the holder. Once the filters were dry, they could then be carefully placed onto the support pad and enumerated by SPC (details of the Chem*Scan* procedure are published in Stevenson et al. (2014)).

The method of using *EasyStain* and SPC has not been published before. Reynolds et al. (1999) used SPC and the "Chem*Scan* Detection of *Cryptosporidium* Oocysts on a Membrane Filter Kit" (bioMérieux), which is no longer available. Zanelli et al. (2000) used SPC to enumerate *Cryptosporidium* oocysts, staining the oocysts with fluorescein isothyocyanate labelled monoclonal antibodies (FITC-mAb), obtained from the same supplier as AES Cheumnex / bioMérieux used for the Chem*Scan* kit. *EasyStain* consists of a mixture of a Cryptosporidium-specific lgG1 mAb and a Giardia-specific lgG1 mAb. Due to the possibility of staining differences, it was necessary to test the reliability of the method, and calibration curves (Figure A1) were produced for enumerating low numbers of oocysts in sterile DI water as well as in the column effluent matrix. The linearity of the regression line in Figure A1 demonstrates that the method of using *EasyStain* and SPC is a reliable enumeration method down to a detection limit of a few cells.

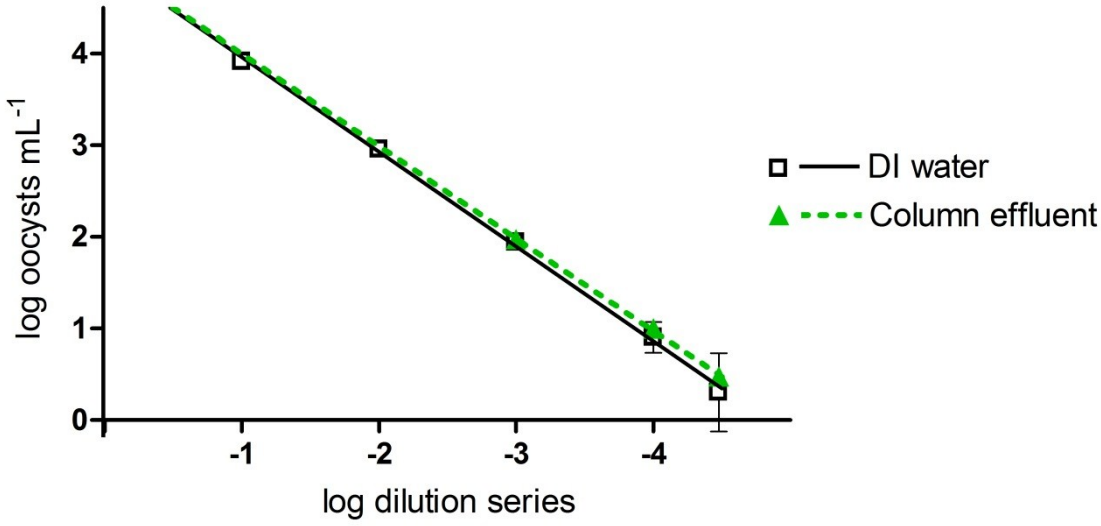

Figure A1: Calibration of enumeration method for *C. parvum*

Enumeration of *C. parvum* oocysts stained with *EasyStain* using Chem*Scan* RDI. Tenfold dilution series of oocysts were counted using sterile deionised (DI) water and the column effluent. Results are presented with a regression line as log means ± standard deviation (3 replicates).

Appendix B

Chapter 2 is based on the publication:

Stevenson, M.E., A.P. Blaschke, S. Schauer, M. Zessner, R. Sommer, A.H. Farnleitner and A.K.T. Kirschner. 2014. Enumerating Microorganism Surrogates for Groundwater Transport Studies Using Solid-Phase Cytometry. Water Air Soil Poll 225. Doi:Artn 1827

The contribution of Margaret Stevenson to this paper was:

- Determination of minimum quantifiable size and maximum filterable volumes
- Enumeration of microspheres using solid-phase cytometry
- Interpretation of results and computation of sample and method limits of detection and quantification

Chapter 3 is based on the publication:

Stevenson, M.E., A.P. Blaschke, S. Toze, J.P. Sidhu, W. Ahmed, I.H. van Driezum, R. Sommer, A.K.T. Kirschner, S. Cervero-Aragó, A.H. Farnleitner and L. Pang. 2015. Biotin- and Glycoprotein-Coated Microspheres as Surrogates for Studying Filtration Removal of Cryptosporidium parvum in Granular Limestone Aquifer Media. Appl Environ Microbiol. Doi:10.1128/AEM.00885-15.

The contribution of Margaret Stevenson to this paper was:

- Performing column tests in the laboratory, including sieve analysis and preparation of influent solutions and aquifer material
- Enumeration of microspheres using microscopy and solid-phase cytometry
- Zeta potential measurements
- Interpretation of results using colloid filtration theory and analysis of breakthrough curves

Chapter 4 is based on the publication:

Stevenson, M.E., R. Sommer, G. Lindner, A.H. Farnleitner, S. Toze, A.K. Kirschner, A.P. Blaschke and J.P.S. Sidhu. 2015. Attachment and Detachment Behavior of Human Adenovirus and Surrogates in Fine Granular Limestone Aquifer Material. J Environ Qual 44: 1392-1401. Doi:10.2134/jeq2015.01.0052.

The contribution of Margaret Stevenson to this paper was:

- Performing column tests in the laboratory, including sieve analysis and preparation of influent solutions and aquifer material
- Enumeration of nanoparticles using microscopy
- Zeta potential measurements
- Interpretation of results using colloid filtration theory and analysis of breakthrough curves
- Modelling of breakthrough curves using the commercial software HYDRUS-1D