Doctoral Thesis

Scale Aspects of Microbial Transport and Removal in Groundwater

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by
Thomas James Oudega, M.Sc.
[redacted]

Supervised by:
Univ.Prof. Dipl.-Ing. Dr.techn. Alfred P. Blaschke
Institute of Hydraulic Engineering and Water Resources Management
TU Wien

Univ. Prof. PD. Mag. Dr. Andreas H. Farnleitner, MSc. Tox.
Institute of Chemical, Environmental and Bioscience Engineering
TU Wien

Examiners:
Univ.Prof. Dipl.-Ing. Dr.techn. Matthias Zessner-Spitzenberg
Institute of Water Quality and Resource Management
TU Wien

Prof. Dr. Jack F. Schijven
Department of Earth Sciences - Environmental Hydrogeology
University of Utrecht

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Summary

Groundwater is an important source of drinking water around the world; therefore, its microbiological quality is of the highest importance to human health. The focus of this doctoral thesis is on the transport and fate of microorganisms in groundwater meant for drinking water production.

One of the greatest challenges involved in the process of providing safe drinking water is the assessment of the presence and concentrations of waterborne pathogens in the source water, i.e. groundwater. Measuring this is laborious and expensive and thus, cannot be done continuously. Often, pathogen concentrations are too low to detect, but can still be at unacceptable levels for public health. Because of this, it is important to use groundwater that has been sufficiently filtered through the porous media of the subsurface to reduce concentrations of waterborne pathogens sufficiently. However, the distance that groundwater should travel before it can be considered safe depends on the type of pathogen in addition to the geochemical composition of the aquifer. Because of these variations in aquifer composition, ideally, aquifers should be tested separately for their capacity to remove microorganisms from groundwater. A good way of performing such testing is to use tracer tests with microbial surrogates. Unfortunately, this is not always possible because of the practical difficulties involved. Furthermore, microbial surrogates do not necessarily accurately model the target pathogen in every material, and since pathogens commonly cannot be used in the field, column tests are often used to compare the surrogate to the pathogen. For these reasons, laboratory column tests are often done instead of field tests. However, these tests are performed on a much smaller scale; therefore, methods need to be developed to upscale the results based on the actual conditions of the field site. After obtaining the correct removal capacity of the aquifer, the minimum groundwater protection zone around a drinking water pumping well needs to be calculated. One method for performing this calculation is to define a health-based target of an acceptable risk (infections/person/year), and to perform a quantitative microbial risk assessment (QMRA)

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using different amounts of microbial removal, which can then be translated to microbial transport distances to select a suitable groundwater protection zone. Another option is to look at unconventional methods for measuring microbiological groundwater quality, which could help us in certain cases, for example at remote locations where a microbiological laboratory is not readily accessible. The goal of this doctoral thesis is to explore the transport and fate of microorganisms in groundwater at different scales, by answering the following questions: (1) What are the most important parameters and processes that influence upscaling of microbial transport in porous media? (2) How does the size of well protection zones change when moving from one calculation method to another, and what influence does pumping rate play? (3) How could alternative methods for the detection of potentially adverse bacteria be used to help with providing safe drinking water?

Following the Introduction, Chapter 2 describes the use of field and column tracer tests to investigate the processes involved in subsurface microbial transport. It was found that subsurface heterogeneity and how much it conduces preferential flow were the most important factors affecting upscaling of microbial transport, and were more important than the size or type of microbial tracer. Chapter 3 describes the calculation of safe setback distances in a well field near Vienna, Austria for two pathogens, namely *Cryptosporidium* and *Campylobacter*. According to our best estimates, specifically for this study site and only for these two pathogens, it was found that the groundwater protection zone did not significantly change in size when switching from the 60-day travel time method to the QMRA method (using an acceptable risk level of 10^{-4} infections/person/year). However, QMRA needs many input parameters that are difficult to measure accurately, and relying on literature values instead of accurate measurements can lead to larger setback distances. Chapter 4 deals with a semi-quantitative, easy-to-use microbiological test called the Laboratory Biological Activity Reaction Test (LAB-BART) that tests for metabolic activity of potentially adverse bacteria in groundwater, which was applied at two riverbank filtration sites in Austria in conjunction with conventional methods used to assess the chemical and microbiological groundwater quality. Although these tests are not as accurate as other methods, such as polymerase chain reaction (PCR), it was found that LAB-BART can serve an important purpose in terms of pre-screening waters of interest in remote locations, or as

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indicators of potential changes in groundwater quality due to any changes in the flow field, especially when problems with well clogging or biofouling arise.

In this doctoral thesis, further insight was gained into the processes and parameters influencing microbial transport and how this knowledge can be used to define safe groundwater protection zones for drinking water production. Results based on our work imply that there is a large difference between microbial transport at different scales, even at the same location, and a thorough understanding of different processes (including preferential flow) in the hydrological system is necessary to ensure microbially safe drinking water from groundwater.

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Zusammenfassung

Weltweit stellt das Grundwasser eine essenzielle Trinkwasserquelle dar, weshalb ein hygienisch einwandfreier Zustand von höchster Relevanz für die menschliche Gesundheit ist. Diese Doktorarbeit konzentriert sich auf Untersuchungen, die zu einem besseren Verständnis der Prozesse beim Transport von hygienisch relevanten Wasserinhaltsstoffen und damit zur Verbesserung eventuell erforderlicher Maßnahmen zur Sicherung von Trinkwasserressourcen beitragen.

Eine der bedeutendsten Herausforderungen im Streben nach sicherem Trinkwasser liegt in der Untersuchung von Krankheitserregern, welche durch Wasser übertragen werden. Die Messung ihrer Konzentrationen gestaltet sich als aufwendig und kostenintensiv, was eine kontinuierliche Überwachung erschwert. Häufig sind die Konzentrationen dieser Krankheitserreger zu niedrig, um von Standardmethoden erfasst zu werden. Dennoch können sie unbemerkt ansteigen und Werte erreichen, die die menschliche Gesundheit gefährden. Daher ist es sinnvoll Grundwasser für die Trinkwasserversorgung zu nutzen, welches durch die Filterwirkung bei der Untergrundpassage im Aquifer eine Reinigung hinsichtlich hygienisch Relevanter Stoffe aufweist. Dies reduziert das Risiko einer Erkrankung bei den Konsumenten des Trinkwassers . Die notwendige Distanz, die das Wasser zurücklegen sollte, um als sicher betrachtet zu werden, hängt von der Art des Krankheitserregers und der geochemischen Zusammensetzung des Grundwasserleiters ab. Aufgrund der unterschiedlichen Untergrundverhältnisse der Grundwasserkörper ist es wichtig, vor einer Trinkwassernutzung den Grundwasserleiter auf seine Fähigkeit zur Entfernung von Schadstoffen zu testen. Die effektivste Methode zur Durchführung solcher Untersuchungen besteht in der Verwendung von Tracertests, welche im Fall von Mikroorganismen mit spezifischen, für die Grundwasserqualität harmlosen, Ersatzstoffen durchgeführt werden. Dies ist aufgrund der oft schwierigen Rahmenbedingungen nicht immer realisierbar. Zudem können diese Ersatzstoffe nicht zwingend die im Fokus stehenden Krankheitserreger (Zielpathogen) in jedem Material ausreichend gut nachbilden. Da

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krankheitserregende Mikroorganismen im Gelände nicht verwendet werden können, werden als Ersatz meist die Untersuchungen in Säulenversuchen durchgeführt, um das Verhalten der Ersatzstoffe mit jenen der Zielpathogen vergleichen zu können. Diese Labor-Säulenversuche werden oft als Ersatz für Feldversuche verwendet, dabei allerdings in einem deutlich kleinerem Maßstab durchgeführt. Aus diesem Grund müssen Methoden entwickelt werden, um die auf Säulenversuche basierenden Ergebnisse auf die tatsächlichen Bedingungen am Feldstandort zu skalieren. Nach Bestimmung der abgeleiteten Entfernungsleistung des Grundwasserleiters, kann die minimal im Grundwassers erforderliche Fließstrecke für eine ausreichende Entfernung der Pathogenen berechnet werden. Eine Methode hierfür ist eine quantitative mikrobielle Risikobewertung (QMRA), bei der ein gesundheitsbezogenes Ziel definiert wird. Eine andere Möglichkeit besteht darin, zur Durchführung mikrobiologischer Bewertungen des Grundwassers einfachere, jedoch meist weniger zuverlässige Methoden, zu verwenden. Dies ist insbesondere bei abgelegenen Standorten, an denen ein mikrobiologisches Labor nicht zur Verfügung steht, eine mögliche Vorgehensweise. Das Ziel dieser Doktorarbeit besteht darin, den Transport und das Schicksal von Mikroorganismen im Grundwasser auf verschiedenen Skalen zu erforschen, indem die folgenden Fragen beantwortet werden: (1) Welche sind die wichtigsten Parameter und Prozesse, die die Hochskalierung des mikrobiellen Transports in porösen Medien beeinflussen? (2) Wie verändert sich die Größe von Brunnen-Schutzzonen bei Wechsel von einer Berechnungsmethode zur anderen, und welchen Einfluss hat die Pumpenrate? (3) Wie könnten alternative Methoden zur Detektion potenziell schädlicher Bakterien zur Unterstützung der Bereitstellung von sicherem Trinkwasser verwendet werden?

Nach der Einleitung beschreibt Kapitel 2 den Einsatz von Feld- und Säulen-Tracerstests zur Untersuchung der Prozesse, die am mikrobiellen Transport im Untergrund beteiligt sind. Dabei wurde festgestellt, dass die Heterogenität des Untergrunds und ihr Einfluss auf bevorzugte Fließwege der zentrale Faktor ist, der bei der Hochskalierung der Ergebnisse aus Laborversuchen auf Feldmaßstab beachtet werden muss. Die Heterogenität des Untergrundes erweisen sich als bedeutender Faktor als die Größe oder die Art des verwendeten mikrobiellen Tracers.

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In Kapitel 3 werden am Beispiel von zwei pathogenen Mikroorganismen (Cryptosporidium und Campylobacter) die für diese Organsimen notwendigen Trinkwasserschutzzonenen eines Brunnenfeldes untersucht. Die Berechnungen zeigen, dass die Größe der Schutzzonen für einen Brunnen bei der Anwendung einer 60 Tage Aufenthaltszeit im Vergleich zu deren Bestimmung mit einer Berechnung nach der QMRA-Methode (Quantitative mikrobielle Risikoabschätzung) kaum Unterschiede ergab. Allerdings erfordert die QMRA Methode eine Vielzahl an Eingangsparametern, die schwierig zu bestimmen sind und man daher häufig auf Literaturwerte anstelle von genauen Messungen zurückgreifen muss. Dieser Umstand kann auch zu einer Ausweisung größeren Flächen für das erforderliche Trinkwasserschutzgebiet führen.

Das Kapitel 4 beschäftigt sich mit einem halbquantitativen, leicht anwendbaren mikrobiologischen Test namens "Laboratory Biological Activity Reaction Test" (LAB-BART), der in Verbindung mit konventionellen Methoden zur Einschätzung der chemischen und mikrobiologischen Grundwasserqualität an zwei Uferfiltrationsstandorten getestet wurde. Trotz der Tatsache, dass diese Tests nicht die gleiche Genauigkeit wie etablierte Methoden, wie die Polymerase-Kettenreaktion (PCR) aufweisen, wurde festgestellt, dass „LAB-BART“ Tests eine wichtige Funktion im Vorab-Screening von für die Trinkwasserversorgung interessanten Gebieten, speziell jene an entlegenen Orten, haben kann. „LAB-BART“ Tests können auch als Indikator für mögliche Veränderungen in der Grundwasserqualität dienen, die aufgrund von Veränderungen der Grundwasserströmung auftreten können, insbesondere wenn es zu Problemen wie Brunnenverstopfungen oder Biofouling kommt.

In dieser Dissertation wurde ein umfassenderes Verständnis für die Prozesse und Parameter gewonnen, die den mikrobiellen Transport beeinflussen. Dieses Wissen bildet eine wichtige Grundlage für die Festlegung von sicheren Schutzzonen für Trinkwasserentnahmebrunnen. Die Erkenntnisse unserer Forschungsarbeit legen nahe, dass bedeutende Unterschiede im mikrobiellen Transport in unterschiedlichen Maßstäben existieren, auch am gleichen Standort, und dass daher ein tiefgreifendes Verständnis der vielfältigen Prozesse (insbesondere bevorzugte Fließwege) im hydrologischen System unerlässlich ist, um eine ausreichende Sicherheit bei der Trinkwassergewinnung zu gewährleisten.

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1 Introduction

Microbiological drinking water quality is of utmost importance to society as substandard quality has been shown to be a hazard to public health (World Health Organization, 2017a). However, microbiological assessment of drinking water is challenging. Because the types and levels of pathogenic bacteria that may be present are not always known, fecal indicator bacteria, such as *Escherichia coli*, are measured to indicate whether a fecal source is present, which may contain pathogens. Even so, waterborne pathogens may still be present even in the absence of *E. coli*; therefore, drinking water that is deemed safe because of the absence of fecal indicators might in actuality still harbor pathogens that, when ingested, may lead to infections (Foppen and Schijven, 2006; Medema et al., 2003).

Because pathogens are not monitored directly, it is difficult to trace water-borne disease outbreaks back to the water from a particular pumping well, and it is often not known that the outbreak originated in drinking water at all (Hrudey and Hrudey, 2019). This can lead to the idea that disease outbreaks originating in drinking water are a thing of the past in the Western World. Unfortunately, this is far from the truth. National surveillance systems have reported a total of 175 waterborne disease outbreaks in the Nordic countries between 1998 and 2012 that affected over 80,000 people (Guzman-Herrador et al., 2015), and 32 drinking water-associated outbreaks in the United States (US) from 2011 to 2012 that accounted for over 400 cases of illnesses and 14 deaths (Beer et al., 2015). Many more outbreaks could have occurred that remained unnoticed or undocumented, especially in developing areas. Overall, it is estimated that every year 2–5 million people die from waterborne, mostly gastrointestinal, diseases originating in drinking water from all sources (Bain et al., 2014; Gleick, 2002).

To prevent water-associated infections, it may be necessary to treat the drinking water before it exits the tap. However, while technical solutions such as chlorination, UV radiation or ozonation exist, these can be hazardous to human health when used in high amounts, and

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are not necessarily a reliable treatment against all pathogens. Furthermore, if the source water has large microbiological quality fluctuations, waterborne pathogens may still end up in drinking water and lead to infections despite a large reduction in their concentration. Therefore, groundwater filtration is an important pre-treatment step for three reasons: (1) it works automatically and at all times, (2) it is cost-effective, and (3) it can be implemented almost anywhere in the world (Tufenkji et al., 2002).

Risks are apparent in using groundwater as a source for drinking water, because any industrial or human/animal activity close to the drinking water wells can also lead to high concentrations of pathogens. Thus, when constructing a drinking water production well, it is essential to select a location sufficiently distant from sites of potential contamination and to define adequate protection zones in which industry and other activities are limited (Blaschke et al., 2016; Schijven et al., 2006a). The size of these zones is the most important consideration because the groundwater needs sufficient travel distance through the subsurface to produce an adequate reduction of potential pathogenic concentrations. In many countries, including Austria, Germany, and Denmark, this distance is defined as a 50- or 60-day travel distance (PDP, 2018). However, considering that outbreaks still occur, this might be a oversimplification in the way to calculate the required travel distance; therefore, we need to gain a better understanding of the transport and fate of microorganisms in groundwater.

The Netherlands is now using an approach in which the groundwater protection zone delineations around drinking water wells are based on a quantitative microbial risk assessment (QMRA). Since the risk of waterborne outbreaks can never be zero, the World Health Organization (WHO) has defined the acceptable risk to be lower than one drinking water-associated infection per 10^4 persons per year. This should be the health-based target that should be aimed for with drinking water production (World Health Organization 2006), and is part of Dutch legislation (Rutjes, Berg, and Schijven 2021). Furthermore, the WHO has stipulated that in the future, risk assessments should be used to further improve the health protection of European citizens (World Health Organization 2017). However, achieving this goal can lead to concerns for policy makers as older well protection zones might suddenly

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increase in size when they are recalculated using a different method. These concerns are justified as it has been found that for Dutch groundwater wells in shallow, unconfined aquifers, a 60-day travel distance is insufficient to reach the health-based target, and that 6–12 times this distance is required (1 to 2 years travel time) (Schijven et al., 2006a).

When performing QMRA, a large amount of data is needed. One of the most important pieces of information is the pathogen removal rate from groundwater during subsurface flow. This rate is sometimes defined as a spatial removal rate (λ in log/m), as an attachment rate coefficient (K_{att} in hr^{-1}) in attachment/detachment models, or as α, which is a unitless parameter that defines the attachment rate as described in Colloid Filtration Theory (CFT) (Pang 2009). This theory states that colloidal particles attach to aquifer grains in a two-step process, namely collision followed by attachment, which can be calculated based on α in combination with other parameters, such as flow velocity, porosity, grain size, and colloid size among others (Tufenkji, 2007). The problem is that in many places around the world, thorough understanding of the hydrological system is lacking; therefore, most of these parameters are unknown. To combat this lack of understanding, tracer tests can be carried out using microbial surrogates that behave similar to common pathogens, with the goal of determining how much reduction of pathogen concentration takes place in the subsurface before the water is pumped up. If this reduction is found to be lacking, additional treatment strategies can be implemented after pumping. Unfortunately, carrying out such tests is not without complications: (1) it is very time consuming and expensive, (2) cooperation of hydrologists and microbiologists is needed, and (3) to inject even non-pathogenic microbial surrogates into the environment, governmental permission has to be obtained, which can be a long and intensive process. Column tests provide an alternative to tracer tests in the outside environment. These tests are cheaper and easier to perform; however, since these tests are done on a much smaller scale, they come with the additional problem of having to upscale results. and reliable upscaling methods are still being worked on. This thesis has the goal of improving our understanding of microbial transport in groundwater by answering the following research questions: (1) What are the most important parameters and processes that influence upscaling of microbial transport in porous media? (2) How does the size of well protection zones change when moving from one calculation method to another, and

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what influence does pumping rate play? (3) How could alternative methods for the detection of potentially adverse bacteria be used to help with providing safe drinking water? (2) How does the size of well protection zones change when comparing distances based on QMRA versus those based on the 60-day travel time, and what influence does pumping rate play? (3) How could alternative methods for the detection of potentially adverse bacteria be used to help with providing safe drinking water?

Chapter 2 describes the essential parameters affecting upscaling of microbial transport in porous media. Previous research has shown that removal rates are much higher in column tracer tests (10-50 cm) than in field tracer tests (>5 m), but the exact reasons for this phenomenon remain unverified (Pang, 2009). As most upscaling research focuses largely on theory and is rarely coupled with field observations, this study included tracer tests with spores of *Bacillus subtilis* and phiX174 coliphages in a 50 cm column as well as in a 25 m long field transect close to Vienna, Austria. A comparison with the available literature indicates how upscaling of microbial transport is affected by various parameters, ranging from size and other characteristics of the microbial tracers to transport distance and type of porous media.

Chapter 3 compares QMRA to the 60 day travel time method for defining the setback distance between a drinking water well and sites of potential contamination. Additional field tracer tests were performed at different pumping rates to allow us to determine the effect of flow rate on the setback distances in the same field site as in Chapter 2. The microbial tracers in these experiments were spores of *B. subtilis*, used in this study as a surrogate for *Cryptosporidium* and *Campylobacter* to study removal by attachment to the porous media. These two pathogens were then considered in the QMRA, carried out to determine the minimum setback distance for a pumping well in an RBF system near the river Danube, to keep the risk of infection below an acceptable level. The resulting setback distances were then compared to the former method of the 60-day travel time to determine if well protection zones would increase in size when switching the method of calculating the protection zone size to QMRA.

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Chapter 4 describes the Laboratory Biological Activity Reaction Test (LAB-BART), an easy-to-use assay that utilizes metabolic capabilities of bacteria to process an array of substrates to semi-quantitatively assess the presence of potentially adverse bacteria in a groundwater sample. Monthly samples were obtained from two RBF systems in Austria along the river Danube for a total period of six months, and were assessed with LAB-BART for iron-related, sulfate-reducing, slime-forming and denitrifying bacteria. Additional measurements for chemical as well as microbiological composition were done to validate the suitability of LAB-BART by identifying relevant bacteria. LAB-BART was examined for its ease of use and accuracy, and to determine in which cases it would be useful.

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2 Upscaling transport of *Bacillus subtilis* endospores and coliphage phiX174 in heterogeneous porous media from the column to the field scale

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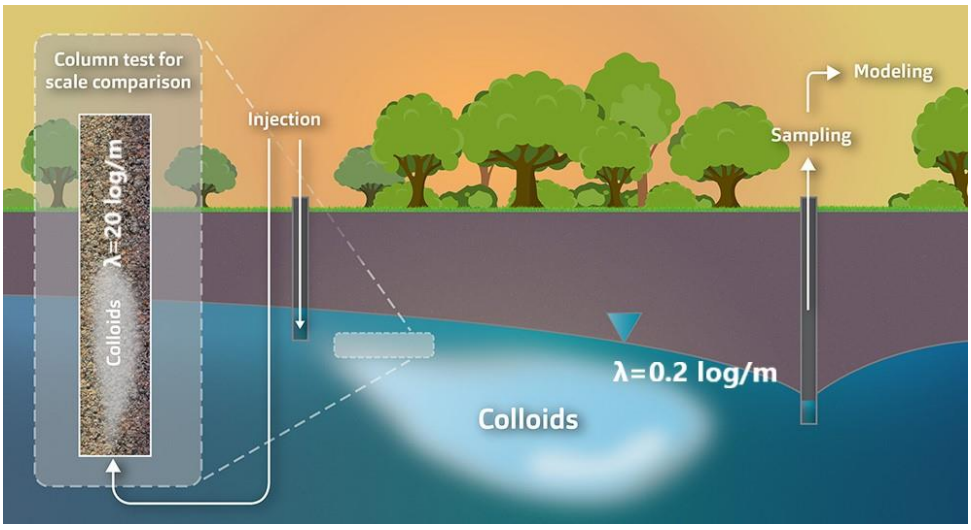
Abstract

Groundwater contamination and transport of viruses and bacteria in aquifers are a major concern worldwide. To estimate an aquifer's capacity to remove pathogens, tracer tests with microbial surrogates are carried out. These tests are laborious and may require special permits, and therefore, column tests are often done instead. Unfortunately, results from column tests tend to grossly overestimate removal rates when compared to the field scale, which can lead to an underestimation of groundwater contamination risks. Scale is an important consideration when examining pathogen transport through porous media, as pathogen removal due to attachment and filtration is rarely a linear process. In this study, field tests were carried out with endospores of *Bacillus subtilis* and coliphage phiX174 over a distance of 25 m in an alluvial gravel aquifer near Vienna, Austria. The sandy gravel material from the field site was also used in column tests with the same tracers. Both attachment-detachment and colloid filtration theory were used to model these test data, as well as log-removal per meter. The results show that the spatial removal rate (log/m) is approximately 2 orders of magnitude higher at the column scale, when compared to the field. A comparison with the literature showed a correlation between the heterogeneity of the porous media and the difference in removal rates between the column and field scale, where larger heterogeneity was associated with a larger difference in removal rates.



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2.1 Introduction

Groundwater is an important source of drinking water for many people around the world. Disease outbreaks due to contaminated groundwater are, therefore, of great concern (Abbaszadegan et al., 2003; Borchardt et al., 2007; Fout et al., 2003). In the last few decades, multiple disease outbreaks across the USA and Europe have been shown to have had their origin in contaminated groundwater, but it is difficult to identify specific risks due to a substantial lack of data (Craun et al., 2010; Guzman-Herrador et al., 2015; Jin and Flury, 2002). It can be assumed that many cases of water-associated infections go undocumented in developing countries as well as in affluent nations (Hrudey and Hrudey, 2019). Furthermore, even though groundwater is still the safer source for drinking water, health risks associated with surface water contamination have been decreasing since the end of last century, and this is not the case for groundwater (Craun, 2012).

One economically advantageous treatment option to reduce the concentration of pathogens in groundwater is to ensure sufficient transport time and distance through the porous media in question. In order to determine the pathogen removal rates in an aquifer, tracer tests with surrogate organisms are often performed (Harvey et al. 1995; Kvitsand et al., 2015; Pang et al., 1998; Wall et al. 2008; Van Der Wielen et al., 2008); however, field tracer tests may require special permits, are time-consuming, expensive and site-specific. Removal of pathogens in the subsurface varies greatly depending on the type of microorganism and its interaction with site-specific aquifer material, so it is often impossible to transfer the results from one site to another (Pang et al., 2009). Soil characteristics, such as chemical attributes, rock fractures, lenses of higher permeability and physical heterogeneity, can negatively influence the removal of pathogens during transport (Harvey et al., 2008; Kvitsand et al., 2015; McMurry et al., 1998). Though it has been shown that preferential transport pathways, if present, may be responsible for decreased pathogen removal rates in porous media, more research is needed to adequately describe microbial transport through such pathways (Zhang et al., 2012).

Although it has been stated that more field tests are crucial for our understanding of removal processes, there is a severe lack of these tests at the field scale (Bradford and Harvey, 2017), and therefore, studies on microbial removal are often done in columns in the laboratory. Unfortunately, observed removal processes in columns may not be

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representative of the field scale, and this method often grossly overestimates microbial removal rates and parameters controlling attachment (Bales et al., 1997; Pang, 2009). Thus, it is essential to understand how scale affects colloidal transport in porous media and to identify the dominant factors that influence the upscaling of transport processes.

Most upscaling research focuses largely on theory, for example, by using different modeling approaches to upscale column results to the field scale. These include stream tube models that mimic preferential flow in the field by aggregating different one-dimensional flowpaths (Bradford et al., 2017; Hilpert and Johnson, 2018; Wang et al., 2014). This promising concept is, however, rarely coupled with field observations. In this study we aimed to compare tests done at the field and column scale using microorganisms of different sizes. The same aquifer material was used at both scales to ensure comparability. Using attachment-detachment, colloid filtration theory as well as log-removal rates calculated per meter, these tests were compared to each other and the literature, in order to gain insight into removal processes at different scales. We hypothesize that one reason average removal rates per meter are higher at the column scale when compared to the field scale is because of soil heterogeneity and the fact that preferential flow elements, such as cracks or lenses of coarser material, are not captured at the column scale.

2.2 Materials and Methods

Field study site. Field tests were carried out at the Obere Lobau test site located near Vienna, Austria. The site consists of an injection well (P24) and a pumping well (LB13) at a distance of 25 m. The injection well has a diameter of 51 mm, a depth of 14 m, and the well screen is from 8 to 14 m below the ground surface. The pumping well has a depth of 24 m, a well screen from 5 to 23 m depth, and the pump is located at a depth of 21 m.

The study site is located next to the River Danube, but is separated from it by a dam with an internal barrier, and is therefore not under influence of potential river floods. The site consists of alluvial sediments including gravel, sand and clay, overlain by an approximately 2 m deep sandy silt soil. Loose sandy and semi-consolidated gravels occur from 2 m to a maximum depth of 35 m. These gravel layers are interrupted and underlain by thin clayey silt and consolidated layers, intermittent throughout the area. While gravel accounts for the

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largest portion of the aquifer grain size distribution, a notable amount of fine material is present as well (Figure 2.1). The uniformity coefficient C_U (d_{60} d_{10}^{-1}), a measure of uniformity of the soil, equals 38.4 for our test site, and the coefficient of curvature, C_C (d_{30}^2 $(d_{60} \cdot d_{10})^{-1}$), is 1.68. Soils are considered uniform if $C_U < 4$ and C_C is between 1 and 3 and low uniformity ($C_U > 4$) leads to higher dispersivity (Freeze and Cherry 1979). The bulk density of the material is 2.24 g cm^{-3}. The groundwater at the site is iron-rich and anoxic (Table 2.1).

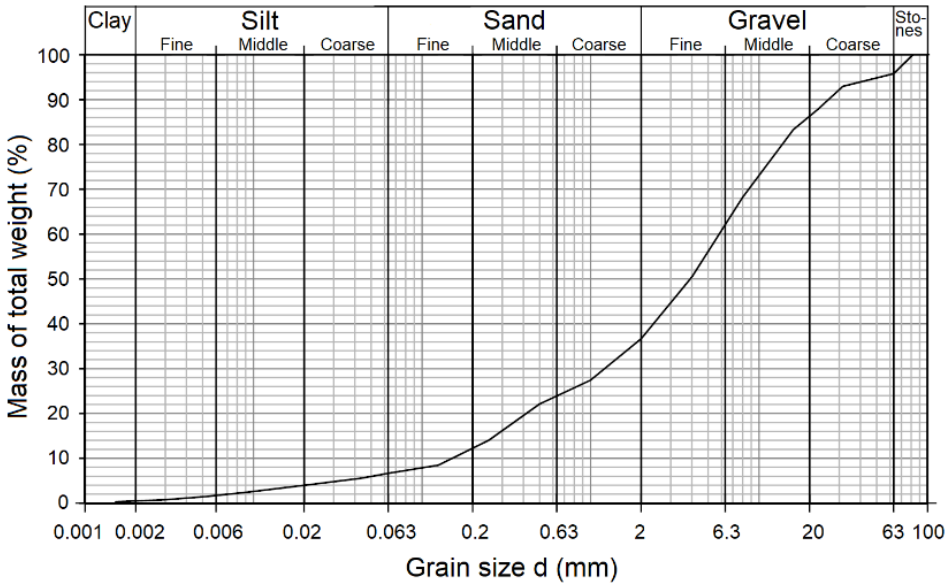

Figure 2.1. Sieve analysis of soil material taken from P24 from a depth of 11 to 12 m

Table 2.1. Chemical analysis of groundwater and Viennese tap water

	Groundwater properties[a]	**Tap water properties**
Water level depth (m)	4.0 - 6.5	-
Groundwater gradient	0.0014	-
pH	7.3	8.0
EC[b] (µS cm^{-1})	637	266
Temperature (°C)	10.5 - 11.2	8.6 - 10.0
Oxygen (mg l^{-1})	0.0 - 0.6	9.7 - 10.3
TOC[c] (mg l^{-1})	1.1	0.4
Iron (mg l^{-1})	2.1	<0.05
Manganese (mg l^{-1})	0.4	<0.02
Chloride (mg l^{-1})	13	2.6
Sodium (mg l^{-1})	9.1	1.2
Calcium (mg l^{-1})	64	46
Kalium (mg l^{-1})	2.0	<0.5
Magnesium (mg l^{-1})	14	9.3

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Sulfate (mg l^{-1})	21	11
Nitrite (mg l^{-1})	<0.01	<0.01
Nitrate (mg l^{-1})	<1	4.9

[a] Measured in wells P24 and LB13, [b] Electrical Conductivity (EC), [c] Total Organic Carbon (TOC).

Tracer preparation and analysis. Sodium bromide (NaBr) was used in each test as a conservative tracer. Restrictions in regards to the concentration of the injected tracers in the field were imposed by the authorities of the City of Vienna and the maximum concentration of NaBr to be injected was 100 mg l^{-1}, and for microorganisms, 10^{12} PFU l^{-1} and CFU l^{-1}, respectively. In the field, bromide samples were taken by an autosampler and stored in plastic test tubes. Analysis was performed at the TU Wien with HP/LC Chromatography (Metrohm ECO IC, Herisau, Switzerland), no longer than two days after sampling. During column tests, electrical conductivity (EC) measurements were carried out by means of a flow-through cell.

Bacillus subtilis is a rod-shaped, gram-positive, aerobic, non-pathogenic bacterium present in low-temperature environments (Wightman et al., 2001). Under non favorable conditions the vegetative bacterium is able to form endospores. Its size was measured with an electron microscope to be 1.5 µm in length and 0.5 µm in width (FEI Company, Hillsboro, USA). The spore's wall has an overall negative charge (Harden and Harris, 1953). The spores of strain ATCC 6633 were provided in freeze-dried form. The day before use, a defined amount of the freeze-dried powder was suspended at room-temperature in deionized water, treated in an ultrasonic bath for about 5 min and thoroughly vortexed to break up any clumps in the suspension before being stored at 4 °C. Pasteurization was used to kill off any leftover vegetative cells of *B. subtilis*, according to ISO 14189:2013(E) (ISO, 2013). This suspension was injected within 3 days of preparation. Samples were stored in glass test-tubes, cooled and brought to the Medical University of Vienna for analysis. Before processing, the samples were heat treated at 70 °C for 10 min to inactivate vegetative cells, no later than 24 h after collection. Incubation was at 36 ± 2 °C for 44 ± 4 h on plate count agar (Tryptone Glucose Yeast Extract, Oxoid, Hampshire, UK). The enumeration was done by counting the colonies formed.

PhiX174 is a single-stranded DNA, non-enveloped, virus with a size of 26 nm and a spherical shape (Deblois and Bayer, 1974; Meder et al., 2013). Its host cell is *Escherichia coli*,

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and it is not pathogenic for humans (Dowd et al., 1998). Even though phiX174 has essentially no charge at near-neutral pH (Dowd et al., 1998; Fujito and Lytle, 1996; Michen et al., 2011), it is generally seen as a good viral surrogate for virus transport, because of its stability and low hydrophobicity (Arkhangelsky and Gitis, 2008; DeBorde et al., 1999; Shields and Farrah, 1987). PhiX174 viruses were prepared following the international standard ISO 10750-2 (ISO, 2002). The virus stock suspension in 10 ml deionized water was mixed with 790 ml tap water for column tests and 1490 ml groundwater for field tests, and injection was performed within 48 h of preparation. The enumeration was done by double layer semi-solid agar overlay method. *Escherichia coli* of strain ATCC 700078 was used as a host and incubation was at 36 ± 2 °C for 18 ± 2 h. The volumes of processed samples were 3 to 9 ml, depending on the presumed concentrations of microorganisms.

The zeta potentials of both colloids were measured by electrophoretic light scattering (Malvern Pananalytical Zetasizer Nano ZSP, UK). The concentrations of microorganisms used for the measurements were the same as during injection (10^6 CFU ml^{-1}). The measurements were done in Lobau groundwater, Vienna tap water, and 10 mM NaCl buffered to both pH 7.3 and 8.0. All background matrices were sterile filtered before the measurements. The measurements were carried out in triplicate.

Field Experiments. Tracer experiments were done in duplicate with spores of *B. subtilis* and phage phiX174, injected separately. The groundwater gradient between P24 and LB13 was approximately 0.02 for all tests, and the difference in gradient between duplicate experiments was never greater than 2.8‰ (or 7 cm). The pumping rate of 5 l s^{-1} was kept constant for at least 72 h before injection to ensure a stable groundwater table. One hour before injection, a sample was taken to verify that there was no background detection of *B. subtilis* or phiX174. A suspension of a microbial tracer was injected with a total amount of $1.65 \cdot 10^{12}$ and $1.16 \cdot 10^{12}$ CFU spores of *B. subtilis* in 1.5 l groundwater, for the first and second test, respectively; and $2.1 \cdot 10^{12}$ and $2.3 \cdot 10^{12}$ PFU phage phiX174 in 1.0 and 1.5 l groundwater, respectively. The suspension was injected in P24 by means of peristaltic pump, in 1 to 2 min. This was immediately followed by an injection of 100 g NaBr in a volume of 1000 l of groundwater by fuel pump, which took approximately 15 min. These injections were done below the water table (to a depth of 7 m) in order to minimize the amount of oxygen added

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to the groundwater. Injection did not affect the water levels in the surrounding piezometers during the tests.

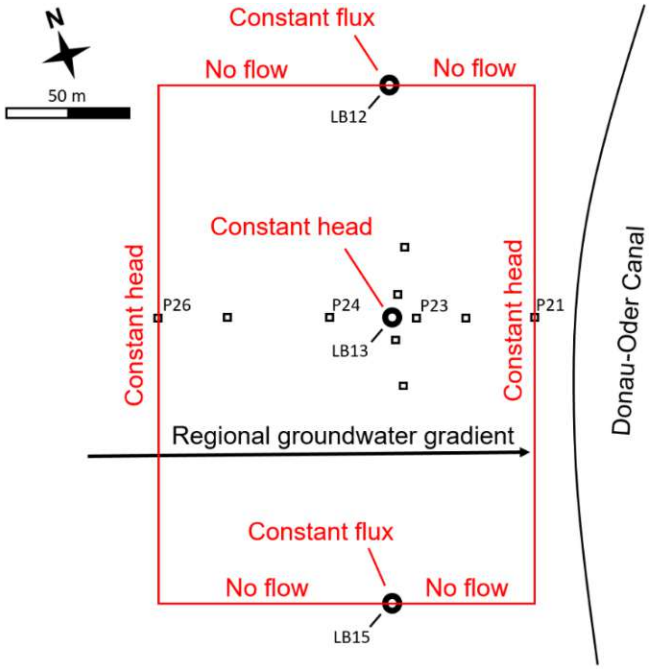

Figure 2.2. Overview of the field site with the model domain and boundary conditions in red. LB wells (circles) are pumping wells, P wells (squares) are piezometers.

Column experiments. The 500 mm long x 70 mm diameter Plexiglas column was freshly packed with new material from the field site for each column test. The material was taken at a depth of 11 to 12 m, either from P24 or from the closest well to the pumping well (P23, Figure 2.2). Stones larger than 5 cm were removed, as these would affect water flow too much in a column with a 70 mm diameter (Weaver et al., 2013), and the ratio of d_{col}/d_{50} (the inner column diameter divided by the effective grain size) was 350, much higher than the recommended minimum ratio of 50, to ensure minimal potential wall effects in the column (Knappett et al. 2008a, Sodré and Parise 1998).

The influent water used in the column tests was standard Viennese tap water, which is Alpine karstic spring water with a pH of approximately 8.0 and an EC of 250 µS cm^{-1}. In contrast to the groundwater of the field site, the tap water was oxic and low in iron (Table 2.1). The columns were rinsed for a minimum of 20 pore volumes (PV) and the experiments were run at a flow rate of 18 ml min^{-1}, which was the highest possible flow rate in the column without a build-up of pressure due to the fine material, causing the tubes to burst off at the connections.

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An 800 ml solution was injected, containing 400 ml with the colloidal tracers prepared separately for the four different tests ($1.08 \cdot 10^9$ and $4.00 \cdot 10^9$ spores of *B. subtilis*; $2.24 \cdot 10^8$ and $2.30 \cdot 10^9$ phages phiX174) and 400 ml tap water with 2 mM l^{-1} NaBr. This was injected over the course of 45 minutes with a peristaltic pump from an automatically stirred Erlenmeyer flask. Duplicate tests were carried out for each tracer. Samples were taken by hand every 2 min, while the EC was measured in a flow-through cell between sampling intervals.

Field Test Modeling. The field tests were modeled with a one-site attachment-detachment model, using HYDRUS 2/3D software (Šimůnek et al., 2016). The three-dimensional domain was defined as a cuboid with a depth of 24 m, as local drillings suggest that an aquiclude exists at this depth, comprising of a thick clay layer. The domain boundaries upstream and downstream were assigned constant heads at P26 and P21, respectively, the furthest points from the pumping well where reliable water level data was available for all tests (Figure 2.2). In the transverse direction, no-flow boundaries were located at the first pumping wells on either side of the flow line, at a distance of 105 m north (LB12) and 152 m south (LB15) of the pumping well, LB13. The pumping wells LB12 and LB15 were assigned constant flux boundary conditions of 7.2 l s^{-1} and 12.5 l s^{-1}, respectively.

The well screen was defined as a cylinder with a diameter of 30 cm. This was modeled as a constant head boundary, in order to have full control over the gradient between the points of tracer injection and extraction. No flow boundary conditions were assigned to the top and bottom of the domain.

Parameter estimation of porosity, hydraulic conductivity and dispersivity in HYDRUS 2/3D was done by fitting, based on water level measurements and conservative tracer tests, carried out with bromide. These values were then used during the subsequent modeling of the microbial transport using the advection-dispersion equation, one-site attachment-detachment model and colloid filtration theory (CFT) equations (Appendix A, Equations A1-3). Due to the fact that our field site has poorly sorted soils, instead of using the grain size d_{50} for the parameter d in CFT (Appendix A, Equation A3.), d_{10} was used. This was considered to be a reasonable assumption because in soils with high amounts of fine material, this size fraction is more important in the removal of colloids than the median size fraction (Martin et al., 1996; Pang et al., 2005; Tufenkji, 2007).

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Column Test Modeling. The column tests were modeled with a one-site attachment-detachment model, with the HYDRUS-1D software package (Šimůnek et al., 2013). The boundary conditions were defined as a constant pressure head on both ends of the column. HYDRUS-1D uses a nonlinear least squares optimization routine, which allows for the inverse estimation of parameters by fitting to observation data. This was used to find values for dispersivity based on the bromide BTC. These values were then used during the subsequent modeling of the microbial transport using the advection-dispersion equation (adapted for one dimension), the one-site attachment-detachment model and the CFT model, as per Equations A1-A3 (Appendix A).

Data analysis. Breakthrough curves of the microbial and conservative tracers were plotted over time and normalized to the initial concentration. From this data, spatial microbial removal rates (λ, log reduction L^{-1}) were calculated for each test as per Equation 2.1, which is valid for three dimensions if the flow is parallel to the x-direction (Kretzschmar et al., 1997):

$$\lambda = \frac{\ln\left(\frac{Q}{N_0}\int_0^{t_f} C(t)dt\right)}{x} = -2.30\frac{log_{10}\left(\frac{Q}{N_0}\int_0^{t_f} C(t)dt\right)}{x} \qquad (2.1)$$

where Q is equal to the flow rate [$L^3\ T^{-1}$], N_0 is the total amount of tracer injected [M], C(t) the concentration at a given time t after injection [$M\ L^{-3}$], t_f is the final time of the test after the pulse has passed through [T], and x the distance from the injection point to the sampling point [L]. The integration was approximated by dividing the time series into sampling intervals, of which it was assumed that the sample concentration was an average value.

2.3 Results and Discussion

Zeta potentials. The zeta potentials for both microbes were measured in 4 different sterile filtered matrices: groundwater sampled from P24 at the field site, Vienna tap water used for the column tests and a standard 10 mM NaCl solution buffered to pH 7.3 and 8.0 using $NaHCO_3$, for literature comparison. The zeta potentials of spores of *B. subtilis* in Lobau groundwater, Vienna tap water and NaCl buffer pH 7.3 and 8.0, were -17.65 ± 0.05, -18.40 ± 1.02, -47.40 ± 0.92 and -30.85 ± 0.45 mV, respectively. For phiX174 the values were -18.47 ± 0.23, -5.15 ± 0.63, -3.27 ± 0.46 and -4.57 ± 2.23 mV. The values measured for spores of *B.*

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subtilis were comparable to other studies, in which values were found of -8 to -15 mV in pH 7, deionized water (Gannon et al., 1991), and -31.5 mV in pH 6.92, groundwater (Pang et al., 2005). In contrast, other studies found zeta potentials of -19 to -10 mV in pH 7.5, 10 mM NaCl (Ahimou et al., 2001), which is less negative than in our study. This may be influenced by the strain of *B. subtilis* used. The zeta potentials of phiX174 were also similar to values in the literature such as -7.5 mV in pH 7 biologically filtered water (Chaudhry et al., 2015), and -8.3 mV in pH 7.3, 154 mM NaCl (Meder et al., 2013). In double-distilled water (ddH_2O) at pH 7, the zeta potential of phiX174 was -31.78 mV, and it became less negative as the pH increased after reaching a minimum around a pH of 5.5 (Chrysikopoulos and Syngouna, 2011). This may explain why phage phiX174 was less negative in Vienna tap water, which had a higher pH than the Lobau groundwater, whereas the zeta potentials of spores of *B. subtilis* were similar in Vienna tap water and Lobau groundwater.

Field test results. The transport and retention behavior of spores of *B. subtilis* and phage phiX174 at the field site is shown in Figure 2.3. The center of mass of both microorganisms breakthrough curve (BTC) precedes the center of mass of the bromide BTC. This is most probably due to pore size exclusion, which traps smaller colloids and dissolved compounds in narrow pore spaces, allowing the center of mass of the pulse of larger colloids to move faster (Pang et al., 1998). Generally, colloidal detachment in all tests was observed to be low. Both BTCs of bromide (Figure 2.3A, B) peaked approximately 8 h after injection. The peak breakthrough concentration of spores of *B. subtilis*, around 5 h after injection, was approximately 2 orders of magnitude higher than that of phiX174, which also peaked 4 to 5 h after injection. Similarly, the percentage of mass recovery of spores of *B. subtilis* was 100 times greater than for phage phiX174 (Table 2.2).

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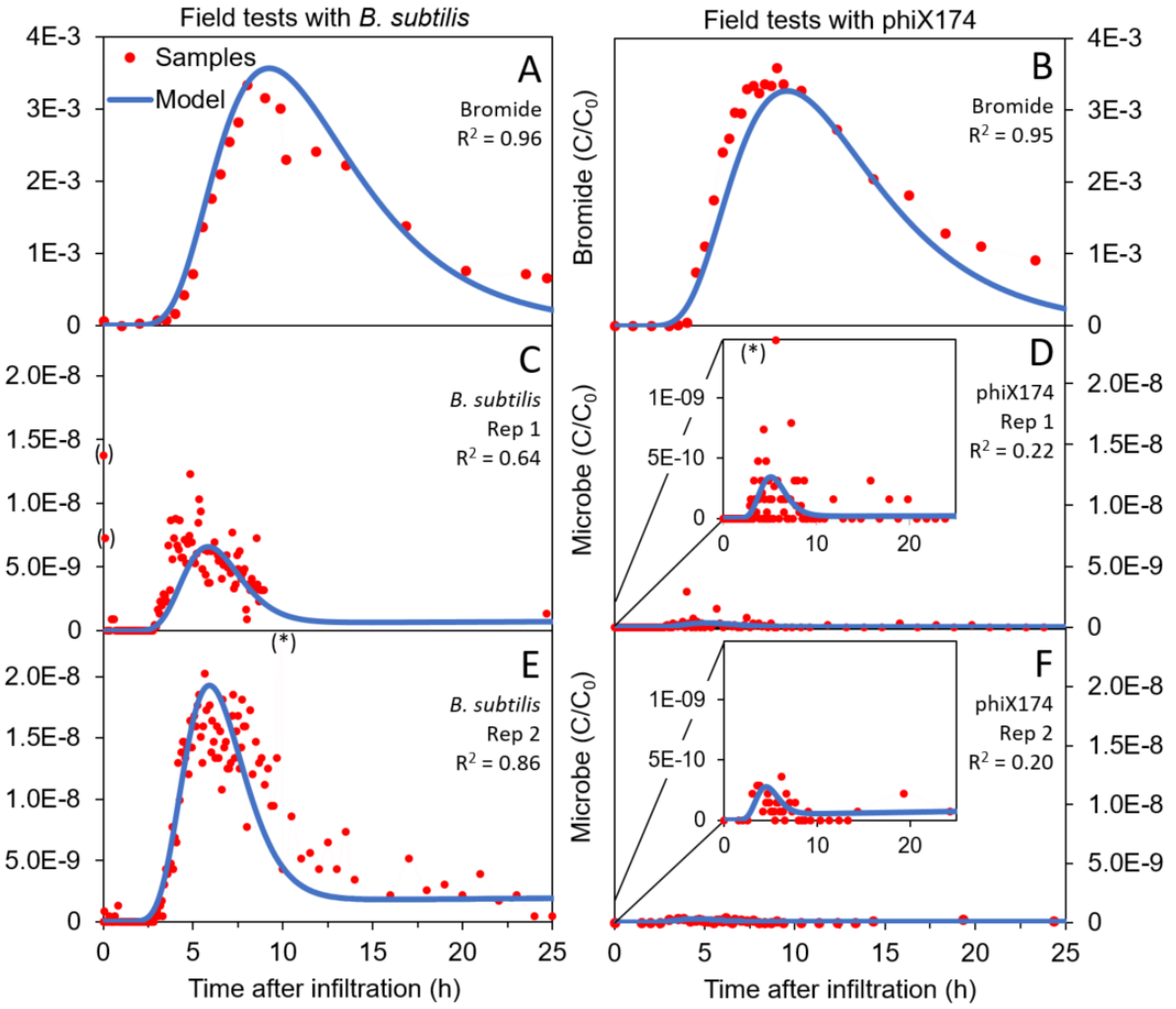

Figure 2.3. Measured samples (red) and modeling results (blue) for the BTCs in field tests with bromide and replicate field tests with spores of *B. subtilis* and phage phiX174 (C/C_0). Samples interpreted as contamination are represented as a red point between brackets. The asterisk (*) stands for samples with a concentration higher than the maximum on the y-axis.

Field test modeling. A hydraulic conductivity of $7.5 \cdot 10^{-3}$ m s^{-1} and a total porosity of 0.12 were calibrated on tests with bromide by trial and error in the one layer model, and are considered realistic for very heterogeneous, coarse gravel (Domenico and Schwartz, 1990). A longitudinal and transverse dispersivity of 1.8 and 0.18 m, respectively, were found to simulate the BTC of the bromide best. The values for porosity and hydraulic conductivity were kept the same for the modeling of spores of *B. subtilis* and phage phiX174. The BTCs of microbial tracers were earlier and less dispersed than the BTCs of bromide. During model calibration a lower dispersivity value for the microorganisms was found (Table 2.2), which indicates the presence of pore size exclusion in our tests (Grindrod et al., 1996; Pang et al.,

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2005). The coefficients of determination (R^2) are low for the modeling of phiX174, which is most likely because of the low breakthrough concentrations (Figure 2.3D, F).

Inactivation of spores of *B. subtilis* was not modeled because it is usually insignificant in saturated column studies, as well as field studies (Bales et al., 1993, 1989; Dowd et al., 1998; Redman et al., 1997). The inactivation rate of phiX174 was found to be about 1-log over a course of 12 weeks in groundwater in a gravel aquifer by DeBorde et al. (1998), and was therefore assumed to be negligible on the timescale of this study (1 day). Straining was not considered because for either microbe, the fraction of colloid size to median grain size was lower than 0.0017, and therefore straining should not occur (Bradford et al., 2002a). However, wedging occurs at smaller colloid/grain ratios, so this might be an issue for the spores of *B. subtilis* in our study (Li et al., 2006). Additionally, natural sand is angular and known to lead to a higher collision efficiency (η) than spherical grains (Saiers and Ryan, 2005). For these reasons, removal efficiencies (η·α) are reported, representing both the colloidal collision and resulting attachment, to be able to compare the attachment of phiX174 and *B. subtilis* in the context of CFT.

In the modeled field tests, phage phiX174 exhibited a higher attachment rate K_{att} (h^{-1}) than spores of *B. subtilis* (Table 2.2). The detachment rates coefficients K_{det} (h^{-1}) were similar between the two microbial tracers. According to CFT, sticking efficiencies (α) were 2 to 3 times higher for spores of *B. subtilis* than for phage phiX174, even though their zeta potential were similar for Lobau groundwater. However, collision efficiencies (η) were around 4 times higher for phiX174, owing to Brownian motion due to their much smaller size (Knappett et al., 2008b). This led to slightly higher removal efficiencies (α·η) for phiX174 in our models. A higher removal of viruses compared to bacteria in heterogeneous aquifer material was also observed other authors (Wang, Bradford, and Šimůnek 2013; Weaver et al. 2013). An explanation for this phenomenon might be that PhiX174 in particular is not as conservative as *B. subtilis*. Alternatively, larger colloids might be more likely to be transported quickly through lenses of course material, resulting in less attachment, and smaller colloids are partially dispersed in dead end narrow pore zones, into which the larger colloids cannot enter (Bradford et al., 2004; Bradford and Harvey, 2017; Mallén et al., 2005).

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Table 2.2. Comparison of modeling results between the microbial tracers and column/field scale. In the model of the field tests, the porosity was manually fitted to the bromide BTC. During the column tests, the porosity, as well as the pore water velocity, was measured. Dispersivities, attachment/detachment rates and sticking efficiency were fitted to the microbial BTC for both scales. Standard deviations are given for field tests average pore water velocity, as well as for parameters that were fitted by inverse optimization.

Field Tests	*B. sub.* 1	*B. sub.* 2	PhiX174 1	PhiX174 2
Average pore water velocity (m h^{-1})	2.5 ± 1.3	2.2 ± 1.2	2.2 ± 1.1	2.4 ± 1.3
Peak breakthrough (C/C_0)	$1.09 \cdot 10^{-8}$	$2.03 \cdot 10^{-8}$	$2.91 \cdot 10^{-8}$	$3.62 \cdot 10^{-10}$
Microbial mass recovered (%)	0.1085	0.1645	0.0039	0.0031
First-order removal rate λ (log m^{-1})	0.23	0.21	0.34	0.35
Longitudinal dispersivity D_x (m)	1.2	1.2	1.5	1.5
Transverse dispersivity D_y (m)	0.15	0.15	0.15	0.15
Porosity θ (-)	0.12	0.12	0.12	0.12
Attachment rate K_{att} (h^{-1})	0.95	1.01	1.33	1.73
Detachment rate K_{det} (h^{-1})	$4.0 \cdot 10^{-3}$	$3.0 \cdot 10^{-3}$	$2.0 \cdot 10^{-3}$	$8.5 \cdot 10^{-3}$
K_{att}/K_{det} (-)	$3.4 \cdot 10^{2}$	$3.4 \cdot 10^{2}$	$6.4 \cdot 10^{2}$	$2.0 \cdot 10^{2}$
Collision Efficiency η (-)	$5.70 \cdot 10^{-2}$	$5.87 \cdot 10^{-2}$	$2.11 \cdot 10^{-1}$	$2.00 \cdot 10^{-1}$
Sticking efficiency α (-)	$5.95 \cdot 10^{-4}$	$4.09 \cdot 10^{-4}$	$1.61 \cdot 10^{-4}$	$2.03 \cdot 10^{-4}$
Removal Efficiency α · η (-)	$3.39 \cdot 10^{-5}$	$2.40 \cdot 10^{-5}$	$3.40 \cdot 10^{-5}$	$4.05 \cdot 10^{-5}$
Coefficient of determination (R^2)	0.644	0.859	0.222	0.199
Column Tests	***B. sub.* 1**	***B. sub.* 2**	**PhiX174 1**	**PhiX174 2**
Pore water velocity (m h^{-1})	1.52	1.68	1.17	1.40
Peak breakthrough (C/C_0)	$1.09 \cdot 10^{-8}$	$5.07 \cdot 10^{-6}$	$8.93 \cdot 10^{-6}$	$7.23 \cdot 10^{-5}$
Microbial mass recovered (%)	0.0093	0.0041	0.0014	0.0073
First-order removal rate λ (log m^{-1})	18.55	20.20	22.31	19.04
Dispersivity D_x (cm)	1.91 ± 1.46	2.27 ± 4.54	1.24 ± 0.21	2.48 ± 0.13
Porosity θ (-)	0.187	0.171	0.197	0.184
Attachment rate K_{att} (h^{-1})	50.77 ± 2.00	117.82 ± 5.07	36.56 ± 1.48	40.09 ± 0.70
Detachment rate K_{det} (h^{-1})	$4.8 \cdot 10^{-2} \pm 2.9 \cdot 10^{-2}$	$5.9 \cdot 10^{-2} \pm 2.3 \cdot 10^{-2}$	$7.55 \cdot 10^{-3} \pm 2.77 \cdot 10^{-3}$	$9.16 \cdot 10^{-3} \pm 3.18 \cdot 10^{-3}$
K_{att}/K_{det} (-)	$1.06 \cdot 10^{3} \pm 6.4 \cdot 10^{2}$	$2.00 \cdot 10^{3} \pm 7.8 \cdot 10^{2}$	$4.84 \cdot 10^{3} \pm 3.1 \cdot 10^{3}$	$4.38 \cdot 10^{3} \pm 2.1 \cdot 10^{3}$
Collision Efficiency η (-)	$3.99 \cdot 10^{-2}$	$3.98 \cdot 10^{-2}$	$3.16 \cdot 10^{-1}$	$2.92 \cdot 10^{-1}$
Sticking efficiency α (-)	0.1014 ± 0.0029	0.1861 ± 0.0048	0.0113 ± $6.5 \cdot 10^{-5}$	0.0107 ± $1.1 \cdot 10^{-4}$
Removal Efficiency α · η (-)	$4.05 \cdot 10^{-3} \pm 1.20 \cdot 10^{-4}$	$7.41 \cdot 10^{-3} \pm 1.90 \cdot 10^{-4}$	$3.57 \cdot 10^{-3} \pm 2.10 \cdot 10^{-5}$	$3.12 \cdot 10^{-3} \pm 3.20 \cdot 10^{-5}$
Coefficient of determination (R^2)	0.040	0.007	0.785	0.934

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Column test results. In contrast to the field test results, the breakthrough of bromide and phage phiX174 were approximately at the same time in the column tests (Figure 2.4B, D, F). Surprisingly, the breakthrough of the spores of *B. subtilis* was much earlier, even arriving in the first samples taken, which is most likely due to sample contamination (Figure 2.4A, C, E).

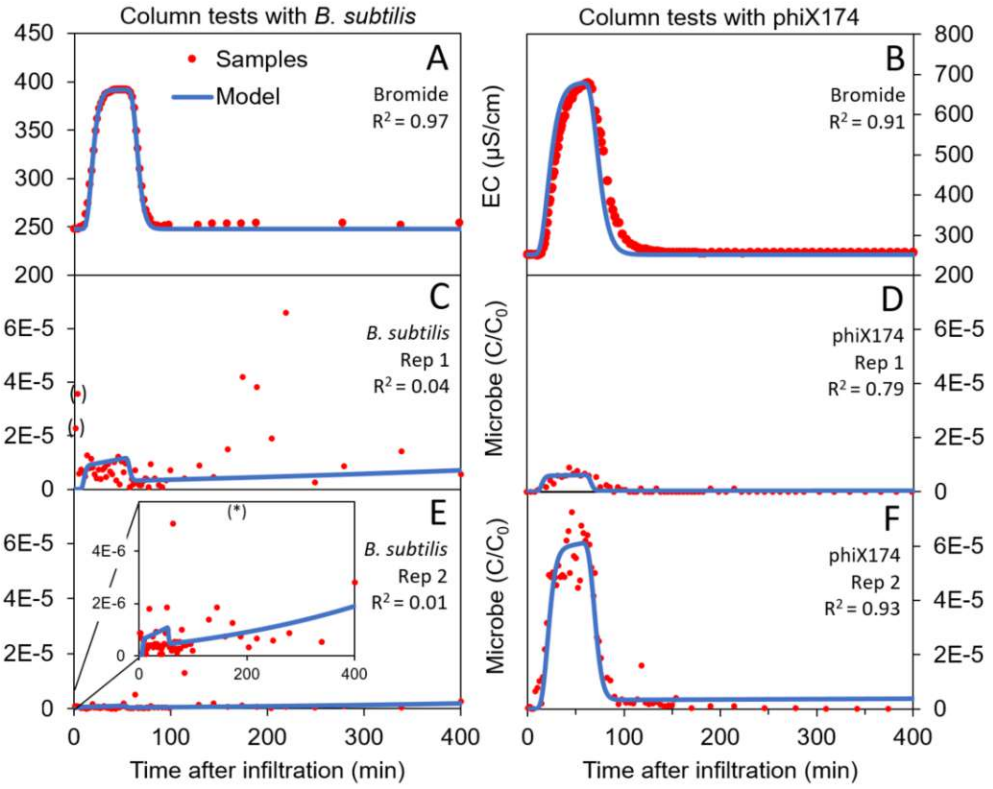

Figure 2.4. Measured samples (red) and modeling results (blue) for the BTCs in column tests with bromide (µS cm^{-1}) and replicate column tests with spores of *B. subtilis* and phage phiX174 (C/C$_0$). Samples interpreted as contamination are represented as a red point between brackets. The asterisk (*) stands for samples with a concentration higher than the maximum on the y-axis.

As seen in the field tests, removal rates in the columns were lower for *B. subtilis* compared to phiX174 (Table 2.2), leading to a higher mass recovery. In both column tests with *B. subtilis*, there was high detachment after the initial peak concentration. Unfortunately, this could not be modeled correctly because the detachment concentrations were too erratic, which led to very low coefficients of determination (R^2) for *B. subtilis* (Figure 2.4C, E).

Column test modeling. Using Hydrus-1D, it was found that both K_{att} and K_{det} were higher for *B. subtilis*, compared to phiX174. Sticking efficiencies (α) were around one order of magnitude higher for *B. subtilis* than for phiX174. Even so, the removal efficiencies (α·η) were similar for both microbes, because of a higher collision efficiency (η) of phiX174. This

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lead to a similar recovery of *B. subtilis* and phiX174 (Table 2.2). The high and irregular detachment of *B. subtilis* lead to high concentrations in the outflow, even after the initial peak had passed. This was not observed in the tests with phiX174, which might be explained by the difference in zeta potential; phiX174 was less negative in Vienna tap water than *B. subtilis*. More negatively charged colloids generally have a higher breakthrough due to higher electrostatic repulsion with negatively charged porous media (Stevenson et al., 2015). In Lobau groundwater, the two microbes have similar zeta potentials. It may be that, for this reason, detachment looks similar in the field BTCs for both microbes.

Upscaling of modeled parameters. The removal rate (λ) for *B. subtilis* in our field study was higher than found by others in similar soils (Pang, 2009; Sinton et al., 2000). The gravel material from our field site has a high fraction of fine material, which may be the reason for this difference (Bradford et al., 2002a; Stevik et al., 2004). Alternatively, the different strains used in the other studies (e.g. strain JH1) might be why our λ was higher, for example because our strain had a less negative charge. In contrast, removal rates (λ) in this study were similar to those found in tests done with other bacteria of a similar size, such as *E. coli* (Pang, 2009; Van Der Wielen et al., 2008). For phiX174, we found similar K_{att} and α values, compared to other studies with phiX174, both on the field and column scale (Van Der Wielen et al., 2008; Wang et al., 2013).

In our study we looked at the K_{att}/K_{det} ratio at the column scale and compared it to the same ratio at the field scale, as a way to evaluate upscaling effects (Table 2.2). It has been hypothesized that this ratio may be the same at all scales (Schijven and Hassanizadeh, 2000); however, in our study, the ratio K_{att}/K_{det} for both colloidal tracers was higher for the column tests than for the field tests by about one order of magnitude. Thus, a stable K_{att}/K_{det} ratio was not found between the column and field scale. As it was difficult to model the falling limb of the *b. subtilis* BTCs, due to the rapidly changing concentrations, we could not verify with certainty if the ratio K_{att}/K_{det} is scale-dependent, but our results imply that it is. Comparing K_{att} and K_{det} at different scales, we observed that in the column tests, K_{att} was about 2 orders of magnitude higher than in the field, while K_{det} was less than 1 order of magnitude higher than in the column tests. This indicates that both K_{att} and K_{det} are scale dependent, albeit K_{det} to a lesser extent, where the high standard deviations for K_{det} should be noted. Furthermore, it should be noted that the flow rate and oxic conditions were not

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the same between the column and field experiments, and that the short injection pulse in the field tracer experiments might obscure dilution and dispersion effects. Therefore, it is not sure that the differences in removal parameters between scales, were solely due to scaling effects.

As with the K_{att}/K_{det} ratio, it has been argued that α-values might be similar between column and field scales, for short travel distances (Schijven and Hassanizadeh, 2000). This was not found to be the case for this study, which has a travel distance in the field of 25 m, as α was lower by around 2-log in the field. This implies that α is scale-dependent; however, the material at our field site is poorly sorted (C_U=38) and this might influence the results, as CFT was developed for uniform soils (Pang et al., 2005). Therefore, α-values might be similar between the column and field scale for other, more uniform porous media. Furthermore, Vienna tap water is more oxic than the groundwater (Table 2.1). This might lead to precipitation of certain oxides, which would increase attachment (Sadeghi, 2012; Schijven and Hassanizadeh, 2000).

The results show that even though the same material was used, there was more removal and attachment (λ, K_{att}, α) in the columns compared to the field, regardless of which modeling method was used. This upscaling phenomenon of relatively more colloidal removal per distance at the column scale compared to the field is commonly observed, and there are many proposed reasons for this. One of these is that it may be due to the smallest representative elementary volume (REV) (Pang 2009), which could capture heterogeneities, and is therefore dependent on type of aquifer material. In order to explore this phenomenon in more detail, the values of λ in the column and field were compared to other studies that were also performed at both scales in the same porous media. Because it was shown that tailing and detachment was important in our tests, it would have been better to compare the ratio of attachment rates, K_{att}, to the literature. Unfortunately, not many studies that are done in the field and in the column, and in the same porous media, include modelling and attachment rates, and for this reason we have chosen to compare the ratio of λ.

The ratio of removal rates λ, in the column to λ, in the field, or in other words, $\lambda_{column}/\lambda_{field}$, is around 100. Studies done by others that have been carried out with microorganisms on both the column and field scale, mostly using the same material at both scales, seem to

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indicate that the ratio of $\lambda_{column}/\lambda_{field}$ is mostly dependent on the heterogeneity of the subsurface (Fig. 2.5A). Notably, this λ-ratio is never 1 (which means that removal in the column is always significantly higher than in the field), even in completely homogeneous material (Hijnen et al., 2005). This suggests that there is something inherent about the way column and field tests are performed that results in a higher λ_{column}. Smith et al. (1985) showed that a disturbed column had 3-log higher removal of *E. coli* than an undisturbed column of the same size, which indicates that macropore destruction has a strong effect on removal rates. Another difference between the column and the field is the arrangement of grains. Grains are not perfect spheres and are deposited horizontally in nature, in line with the flow direction through the aquifer. During column tests, this flow is vertical and perpendicular to this layering effect, which might influence removal during transport. Lastly, we are comparing columns on the centimeter-scale to flow paths on the meter-scale in the field. Along these flow paths, there may be cracks or lenses of higher permeability that are larger than the column size itself. Therefore, extrapolation from the column to the field scale is problematic because we inherently assume a field site without these elements of preferential flow.

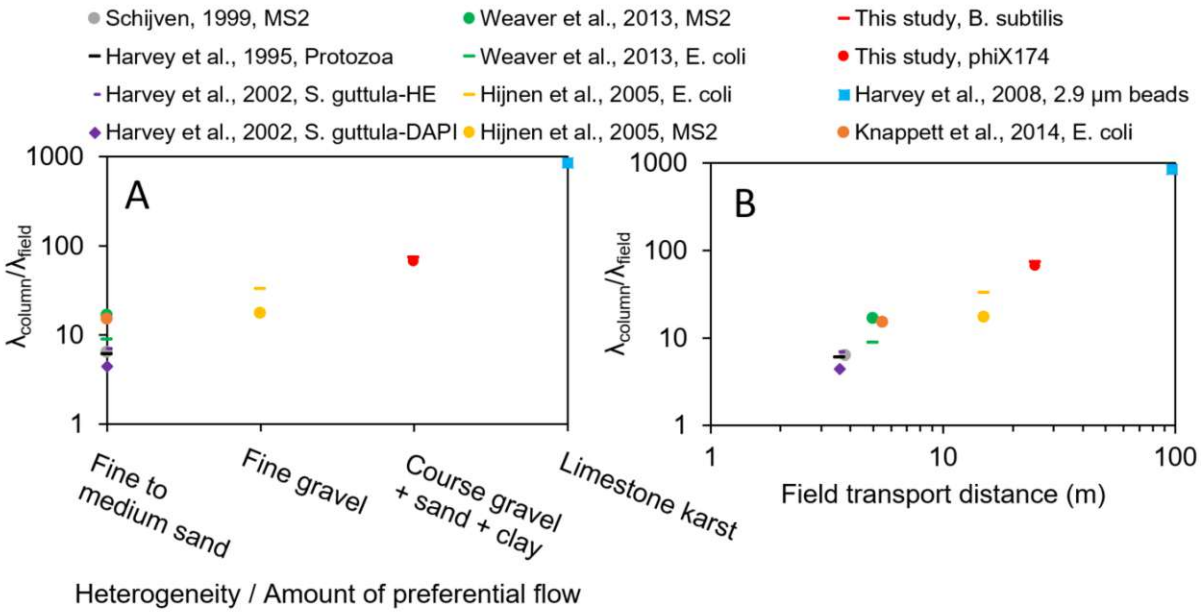

Figure 2.5. (A) Calculated ratios of $\lambda_{column}/\lambda_{field}$ (according to Equation 2.1) from published studies done in columns and field tracer tests in materials of varying heterogeneity and/or varying amounts of preferential flow. (B) The same ratios of $\lambda_{column}/\lambda_{field}$ plotted against the transport distance in the field. Values for λ (log m^{-1}) taken from Pang (2009).

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Figure 2.5B addresses the issue of transport distance (x-axis), which is usually chosen depending on the material type (Fig. 2.5A), to allow for preferential flow paths. This is an attempt at solving the upscaling problem with the use of characteristic lengths, similar to REV, as mentioned previously. It has been suggested that using characteristic lengths is a good way to parameterize the order-of-magnitude of problems, which might lead to enhanced insight into processes at different scales (Blöschl, 2001). In the studies considered, sand aquifers have a $\lambda_{column}/\lambda_{field}$ ratio of around 10 (Fig. 2.5A). Well sorted gravels have a slightly higher ratio (Hijnen et al., 2005; Medema and Stuyfzand, 2020), while poorly sorted gravels (this study) have a ratio of around 100. Karstic aquifers (or other aquifers with extreme preferential flow) can have a λ-ratio of 1000 (Harvey et al., 2008).

The destruction of preferential flow paths due to disturbing the soil when making columns is often cited as a major cause of higher removal rates in columns, because this leads to all matrix flow in the column, and therefore to higher removal rates (Knappett et al., 2011; Smith et al., 1985). Additionally, removal might happen predominantly in the first centimeters after injection, which leads to a decreasing removal rate (λ) with distance (Foppen and Schijven, 2006). This can have multiple explanations, summarized by Pang as being due to straining and heterogeneous/unfavorable attachment (Pang, 2009). A popular theory is that favorable attachment sites are progressively “filled up” and blocked, so that the colloids have to travel further to find an attachment site, causing attachment to be non-linear (Camesano et al., 1999). Additionally, colloidal population heterogeneity might lead to viruses or bacteria with higher sticking efficiencies attaching first, and others later or not at all. This theory of fast versus slow attachment would indicate that the slower attaching colloids in the population would be a minority, since most attachment happens right after injection, and Schijven et al. (2002) found that the chemical heterogeneity of the aquifer material was more important than the heterogeneity of the colloidal population. It is also possible that microorganisms attach to other particles like clay and are co-transported, thereby enhancing their travel distance (Schijven and Hassanizadeh, 2000). Lastly, in sub- or anoxic aquifers with iron-rich groundwater, like the one in this study, iron-oxides might precipitate around the injection well when oxygen is introduced during injection (Teutsch et al., 2005). This might influence removal rates, because iron oxide grain coatings provide sites for enhanced attachment (Ginn et al., 2002; Knapp et al., 1998). We tried to minimize

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oxidation by using a piezometer that was never before used as an injection well, only injecting recently pumped-up groundwater, and making sure injection was always below the water table.

An explanation for the λ-ratio increasing as heterogeneity increases could be that flow through preferential flow paths is faster than fine matrix flow, decreasing attachment, and these preferential flow paths cannot be recreated in the columns. Flow through crack networks can be up to 4 times higher than that of the adjacent matrix (Coppola et al., 2009; Freeze and Cherry, 1979). Other authors have made observations about scaling between field tests, noting the inverse relationship between the length of the flowpath and the removal rate λ (Pang et al., 2005). Longer field flow paths have reduced λ, which increases the $\lambda_{column}/\lambda_{field}$ ratio. The length of the field flow paths considered in the present literature comparison range from 3.6 m to 97 m (Fig. 4B). This could lead to a bias in the comparison of the λ-ratios in Fig. 2.5A. The flow path lengths of these field studies were probably chosen so that it could capture flow elements typical of the aquifer, i.e. crack networks or lenses of course material. Therefore, even though some bias might exist due to transport distance, heterogeneity of the material may be one of the more important parameters affecting the $\lambda_{column}/\lambda_{field}$ ratio based on the literature comparison in Fig. 2.5, and transport distance is usually chosen based on heterogeneity. This upscaling ratio may also be altered depending on type of colloid, as well as differences in ionic strength of the groundwater matrix (Ahimou et al., 2001; Bradford et al., 2002a; Wang et al., 2013), groundwater chemistry, chemical composition of aquifer media, heterogeneity within the microorganism community, to name a few, but in our study these influences were minimal since the same porous media and colloids were used in the column and the field tests.

2.4 Conclusions

Upscaling colloidal transport from the column to the field scale is challenging because of the complex structures often inherent to porous media. These structures create intricate flow patterns which are difficult to quantify, and therefore problematic when upscaling transport processes. In this study, results reveal that column tests overestimate log-removal rates by approximately 2-log in poorly sorted gravel material. Similarly, values for K_{att} and the CFT

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parameter α were overestimated by 1 to 2 log in the column. Preferential flow due to material heterogeneity may be the main driver for this phenomenon, as it is difficult to recreate preferential flow paths in a small column. This needs to be confirmed with more opportunity for comparison with upscaling studies in other types of aquifer materials. Furthermore, we showed that in gravel material, phage phiX174 (as a surrogate for viruses in general) has a higher removal rate compared to spores of *B. subtilis*, possibly because larger colloids (such as *B. subtilis*) are transported more than smaller colloids through preferential flow paths, created by course gravel lenses. This, combined with the fact that the two tracers have similar zeta potentials in Lobau groundwater, and thus phiX174 will have more collisions with the porous media leading to more attachment, could explain the higher removal rate of phiX174 over *b. subtilis*.

The environmental implication of this study is that based on the comparison of our study with literature data, preliminary conclusions surmise that the type of porous media affects the upscaling relationship, mainly due to differences in prefential flow. However, it is not precluded that other important drivers play an important role, such as the type of microorganism or physicochemical conditions in the subsurface. Future research is planned to test the findings of this study, focusing on mesoscale pathogen transport in a large, undisturbed gravel column.

This chapter was based on the following publication:

Oudega, T.J., Lindner, G., Derx, J., Farnleitner, A.H., Sommer, R., Blaschke, A.P., Stevenson, M.E., 2021. Upscaling Transport of *Bacillus subtilis* Endospores and Coliphage phiX174 in Heterogeneous Porous Media from the Column to the Field Scale. Environ. Sci. Technol. 55, 11060–11069. https://doi.org/10.1021/acs.est.1c01892

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3 Transport and removal of spores of *Bacillus subtilis* in an alluvial gravel aquifer at varying flow rates and implications for setback distances

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Abstract

To guarantee proper protection from faecally transmitted pathogen infections, drinking water wells should have a sufficiently large setback distance from potential sources of contamination, e.g. a nearby river. The aim of this study was to provide insight in regards to microbial contamination of groundwater under different flow velocities, which can vary over time due to changes in river stage, season or pumping rate. The effects of these changes, and how they affect removal parameters, are not completely understood. In this study, field tracer tests were carried out in a sandy gravel aquifer near Vienna, Austria to evaluate the ability of subsurface media to attenuate *Bacillus subtilis* spores, used as a surrogate for *Cryptosporidium* and *Campylobacter*. The hydraulic gradient between injection and extraction was controlled by changing the pumping rate (1, 10 l/s) of a pumping well at the test site, building upon previously published work in which tracer tests with a 5 l/s pumping rate were carried out. Attachment and detachment rate coefficients were estimated using a HYDRUS-3D model and ranged from 0.12-0.76 and 0-0.0013 hr^{-1}, respectively. Setback distances were calculated based on the 60-day travel time, as well as a quantitative microbial risk assessment (QMRA) approach, which showed similar results at this site; around 700 m at the highest pumping rate. Removal rates (λ) in the field tests ranged from 0.2-0.3 log/m, with lower pumping rates leading to higher removal. It was shown that scale must be taken into consideration when determining λ for the calculation of safe setback distances.

3.1 Introduction

Waterborne disease outbreaks are still a major health issue worldwide (Beer et al., 2015; Beller, 1997; Rasmuson et al., 2019). To reduce the risk of these outbreaks, pre-treatment steps such as riverbank filtration can be used to lower pathogen concentration (Ray et al., 2002; Sharma et al., 2012). During subsurface flow, the removal of pathogens is influenced by groundwater flow rate, which can vary due to factors such as groundwater abstraction, river stage, seasonal changes, city planning decisions (such as stream rehabilitation) and

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effects of climate change. Furthermore, water level fluctuations can lead to higher concentrations of pathogens (Derx et al., 2013).

In many countries, an adequate removal of pathogens is usually assumed when subsurface travel times are 60 days or more, and setback distances for drinking water wells are often calculated on this basis (Schijven and Hassanizadeh, 2002). However, in contrast to this 60-day time of travel (TOT) approach, the World Health Organization (WHO) has recommended that a quantitative microbial risk assessment (QMRA) approach should be used for defining safe setback distances for drinking water production (World Health Organization, 2017b, 2017a). To be able to carry out a QMRA, reliable transport and removal parameters describing subsurface flow are needed. To accurately quantify these parameters, tracer tests should be carried out at the site of interest, using surrogates that experience similar transport and removal as pathogenic microorganisms.

Studies in columns show that faster flow rates can lead to less removal (Choi et al. 2007; Ryan and Gschwend 1994; Walshe et al. 2010), and attachment and detachment rates have been shown to decrease with decreasing pore water velocity (Bradford et al. 2006; Hendry et al. 1999). An explanation for this is that a lower velocity leads to longer residence times, which increase the probability of microbes to diffuse over the energy barrier, as well as leading to lower hydrodynamic forces, decreasing detachment (Sasidharan et al., 2017), as well as the fact that there is more time for inactivation and/or die-off. Furthermore, Colloid Filtration Theory (CFT) predicts that the sticking efficiency (α) is dependent on the single-collector contact efficiency (η_0), which in turn depends on the flow velocity, because it affects, among other processes, the particle deposition rate due to interception, diffusion and settling (Tufenkji and Elimelech 2004; Yao et al. 1971). This shows that velocity is an important factor affecting bacterial removal in groundwater. However, most tracer tests in the field are carried out within natural (i.e. unforced) gradient flow conditions, which makes it difficult to observe the effects of different flow rates on microbial removal directly (DeBorde et al., 1999; Mallén et al., 2005; Pang et al., 2005). Comparing removal rates at different flow velocities is usually done by comparing tests in different aquifers, and few tests have been carried out with varying flow rates at the same test site (Kvitsand et al. 2015; Pang 2009). Therefore, it is not well understood at the field scale if and how changes in flow velocity affect microbial removal and the parameters that govern it.

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In order to compare setback distances at the various flow rates, a QMRA was carried out for *Cryptosporidium* and *Campylobacter*, as well as calculations of the traditional 60-day TOT. Both pathogens considered in this study are commonly found in human and animal waste, and can cause gastrointestinal illnesses leading to severe and prolonged diarrhea, which may pose significant health risks, especially for vulnerable and immunocompromised patients (Hoogenboezem et al. 2001; Percival and Williams 2013; Teunis et al. 2005, 2002; WHO 2011). *Campylobacter* are fecally borne bacteria that have led to numerous waterborne disease outbreaks in the past years, even in developed countries (Craun, 2012; Guzman-Herrador et al., 2015). *Cryptosporidium* are protozoa, usually present in the form of oocysts which resist degradation, and are generally more persistent in the environment, not unlike spores of *B. subtilis* (Headd and Bradford, 2016). *Campylobacter* is removed more readily, as it is not spore- or oocyst-forming and therefore experiences more die-off and inactivation, processes whereby the organism either dies or is unable to infect (Schijven et al., 2013).

The groundwater flow rates in this study were controlled by changing the pumping rate of an abstraction well in the area, where tracer tests were carried out using spores of *Bacillus subtilis.* The spores of endospore-forming bacteria are very persistent during transport and are therefore regarded as worst-case scenario microbial tracers to study the attachment and filtration of bacteria in the subsurface (Li et al. 2018; Pang et al. 1998; Setlow 1995**, Pang et al. 2005**). *B. subtilis* (~1.5 µm long) is of similar size when compared to many important bacterial pathogens such as *Salmonella spp.*, and is also used as a surrogate for *Campylobacter spp. and Cryptosporidium spp.* (which is up to 7 µm long), even though there are differences in size, surface charge and hydrophobicity (Bradford et al. 2016a; Emelko and Huck 2004; Cools et al. 2003; Chen et al. 2010).

This study builds upon a previous study by Oudega et al. (2021), by performing additional field tracer tests at different pumping rates, which are modeled in HYDRUS-3D to attain parameters values for the transport of the tracers in alluvial aquifers. Furthermore, this study adds a comparison of the 60-day TOT versus the QMRA approach for defining the setback distances from a drinking water well, using *Cryptosporidium* and *Campylobacter* as reference pathogens.

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3.2 Materials and methods

3.2.1 Field site

The study was carried out at the Obere Lobau test site located near the River Danube in Vienna, Austria, and contains alluvial sediments of mostly gravel and sand (d_{50}=4 mm, C_U=38.4). The effective porosity of the material is 0.12 and the hydraulic conductivity is $7.5 \cdot 10^{-3}$ m/s. The groundwater at the site is not in direct contact with Danube river water due to the presence of a dam, and contains little to no oxygen, a near-neutral pH and a high iron content. A more extensive description of the study site, sediment and groundwater characteristics is given in Oudega et al. (2021). The site contains an injection well (P24) and a pumping well (LB13) at a distance of 25 m, which also serves as the sampling point (Figure 3.1). There are additional pumping wells north and south of the study site, which together create a drawdown of hydraulic head and a predominantly west to east groundwater flow. The injection well has a well screen from 8 to 14 m depth, the pumping well has a well screen from 5 to 23 m depth, and both well screens are located fully in the saturated zone.

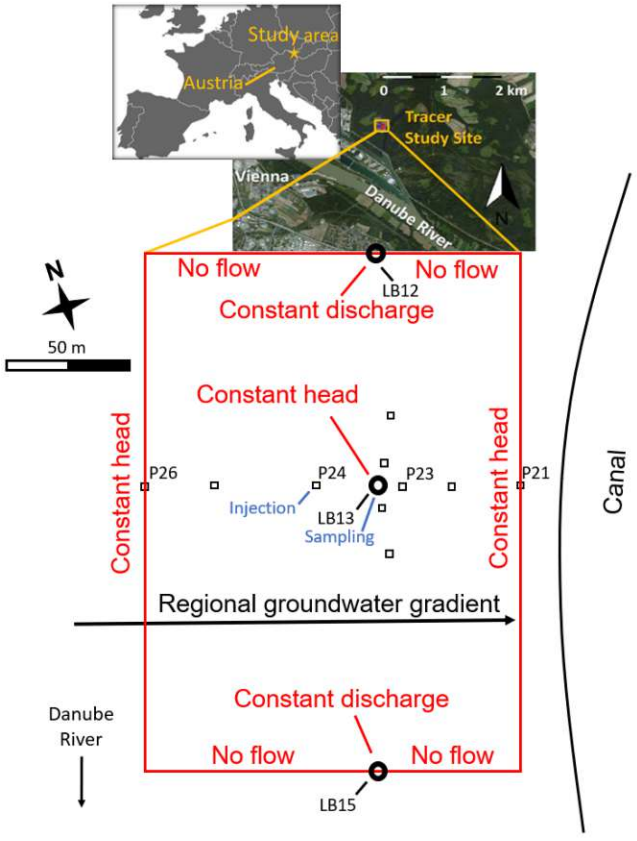

Figure 3.1. Location of the tracer studies conducted in 2018 and 2019. The model domain and boundary conditions are shown in red. Circles (LB) are pumping wells, squares (P) are

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piezometers. The map of the boundary conditions is taken from Oudega et al. (2021), in which the modeling is described in more detail.

3.2.2 Experimental Design

To allow for direct comparison of transport processes at different flow rates, duplicate tracer tests were performed in a forced gradient system with pumping rates of 1, 5 and 10 l/s (Table 3.1). These pumping rates are about an order of magnitude lower than the maximum extraction rates in drinking water wells downstream of the study site. However, due to regulations, higher pumping rates were not allowed. The flow rates given in this table are averages over the transport distance, but not uniform, as they increase towards the pumping well. All experiments were done with spores of *B. subtilis* and a conservative tracer; either uranine or bromide. Tests 5a-b were done with bromide using a pumping rate of 5 l/s (results published in Oudega et al., 2021). The additional tests for this study were carried out with uranine: tests 1a-b with a 1 l/s pumping rate and tests 10a-b with a 10 l/s pumping rate. Test 1pre was carried out with both bromide and uranine to check whether the transport behavior of the two conservative tracers was similar. The absolute difference in hydraulic gradient, which was determined by hand measurements, between duplicate experiments was never greater than 2.8‰.

Table 3.1. Field experiments carried out with spores of *B. subtilis*

Test	Date	Pumping Rate (l/s)	Gradient (-)	Average Flow rate (m/h)	Conservative Tracer	*B. subtilis* injected (CFU/ml)
1pre	06.12.18	1.09	0.0045	0.102	Both	-
1a	07.01.19	1.20	0.0023	0.098	Uranine	$1.33 \cdot 10^9$
1b	18.02.19	0.99	0.0028	0.079	Uranine	$9.13 \cdot 10^8$
5a[a]	24.04.18	5.05	0.0194	0.615	Bromide	$1.10 \cdot 10^9$
5b[a]	28.05.18	4.65	0.0189	0.557	Bromide	$7.73 \cdot 10^8$
10a	21.01.19	9.57	0.0311	1.015	Uranine	$5.64 \cdot 10^8$
10b	28.01.19	9.45	0.0318	0.977	Uranine	$7.87 \cdot 10^8$

[a] Published in Oudega et al. (2021)

3.2.3 Characteristics, culture and essay of the tracers

Bacillus subtilis. All tests were carried out with *B. subtilis* endospores (strain ATCC 6633), which are rod-shaped, aerobic and non-pathogenic bacteria present in low-temperature

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environments (Harden and Harris, 1953; Headd and Bradford, 2016; Wightman et al., 2001). In the spore state, *B. subtilis* bacteria are well protected against damage and survive for a long time (Setlow, 1995; Setlow and Johnson, 2019). Using an electron microscope (FEI Company, Hillsboro, USA), the spores were measured to be 1.5 µm in length and 0.5 µm in width. They have an isoelectric point of pH 2.2 and are strongly hydrophilic (Bradford et al., 2016b; Harden and Harris, 1953).

Preparation and assay was described in Oudega et al (2021). The spores were injected at a concentration of approximately 10^9 spores/ml. The suspension of 1.5 l of groundwater was injected in 1.5 minutes at 7 m depth using a peristaltic pump.

Samples of 12 ml were taken by an autosampler from a flow-through cell linked to pumping well LB13, and stored in glass test-tubes. An initial interval of 5 minutes between samples was used in tests 5a-b and 10a-b, and 60 minutes in tests 1a-b. These intervals were extended after the peaks passed. Depending on the expected concentration, sample volumes of 1, 2, 3, 6 or 9 ml were used on one or up to three petri dishes. The lower the expected concentration, the higher was the sample volume analyzed, using the pour plating technique and PC-Agar (Merck, Darmstadt, Germany).

Bromide. According to the maximum concentration of 100 mg/l NaBr permitted by the government (reference), the bromide (100 g) was injected in a volume of 1000 l groundwater to ensure sufficient concentration in the samples. The bromide solution was injected by a fuel pump 2 minutes after the injection of the *B. subtilis* spore suspension and took approximately 15 minutes. We assumed minimal mixing between the two injection fluids in the injection well, as no groundwater level rise was measured directly after infiltration, and thus the aquifer was shown to be permeable enough to not have mixing inside the injection well. The samples were analyzed at the TU Wien with HP/LC Chromatography (Metrohm ECO IC, Herisau, Switzerland).

Uranine. Uranine was used in tests 1a-b and 10a-b in a concentration of 10 g/l and a volume of 10 l groundwater, using the same injection method as for bromide. The fluorescence of the water was measured directly in the flow-through cell using a GGUN-FL24 flow-through field fluorospectrophotometer (Albillia Co, Neuchatel, Switzerland).

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3.2.4 Modeling

Hydrus.

The subsurface flow was simulated in 3 dimensions with the Richards' equation (Šimůnek et al., 2012):

$$\frac{\partial \theta}{\partial t} = \frac{\partial}{\partial x_i}\left[K(h)\left(K_{ij}^{A}\frac{\partial h}{\partial x_j} + K_{iz}^{A}\right)\right] \qquad (3.1)$$

where θ is the water content (-), t is time (T), x_i is the spatial coordinate (L), K is the hydraulic conductivity (L/T), h is the pressure head (L) and K_{ij}^{A} are components of a dimensionless anisotropy tensor.

The transport of spores of *B. subtilis* was modeled using the following advection-dispersion equation and two-site attachment-detachment model (Schijven and Šimůnek 2002):

$$\frac{\partial C}{\partial t} = D_x\frac{\partial^2 C}{\partial x^2} + D_y\frac{\partial^2 C}{\partial y^2} + D_z\frac{\partial^2 C}{\partial z^2} - v\frac{\partial C}{\partial x} - \frac{\rho_b}{\theta}\frac{\delta S_1}{\delta t} - \frac{\rho_b}{\theta}\frac{\delta S_2}{\delta t} \qquad (3.2)$$

$$\frac{\rho_b}{\theta}\frac{\delta S_1}{\delta t} = k_{att1}C - \frac{\rho_b}{\theta}k_{det1}\,S_1 \qquad (3.3)$$

$$\frac{\rho_b}{\theta}\frac{\delta S_2}{\delta t} = k_{att2}C - \frac{\rho_b}{\theta}k_{det2}\,S_2 \qquad (3.4)$$

where C is equal to the concentration of free spores of *B. subtilis* (M/L^3), D is spatial dispersion (L^2/T), v is the pore-water velocity (L/T, whereby x is the direction of flow), ρ_b is bulk density (M/L^3) and S is the concentration of attached particles (M/L^3). Values for k_{att} and k_{det} (1/T), attachment and detachment rate coefficients, respectively, were found by calibrating the model to the BTC of each test.

The field tests were modeled using HYDRUS-3D software (Šimůnek et al. 2016). The three-dimensional domain was defined as a cuboid with a depth of 24 m, which is the top of a clayey aquitard in the study area. Constant heads were assigned to the domain boundaries upstream and downstream as well as on the main pumping well, as to maintain perfect control over the hydraulic gradient (Figure 3.1). No flow conditions were assigned to the north and south boundaries on either side of the flow line, except for two pumping wells on these boundaries, which were modelled by constant discharge. The values for hydraulic

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conductivity were found by calibration on the basis of the measured water levels in the piezometers during tests. The injection solution was assumed to be mixed in the entire well screen. Estimation of dispersivity values was done by fitting the modeled breakthrough curves (BTCs) of the conservative tracers to the measured data. More details about the model and its parameters can be found in Oudega et al. (2021). To calculate the model-derived 60-day TOT distances (the distance from which the water has to travel for 60 days to reach the pumping well), the modeled hydraulic gradient was extrapolated in space for each test. Darcy's equation was then employed, using the calibrated values for hydraulic conductivity and porosity.

QMRAspot. QMRAspot is a computational tool to analyze and conduct a quantitative microbial risk assessment (QMRA) for drinking water. It was used for this study site to calculate the required removal of *Cryptosporidium* oocysts and *Campylobacter* during subsurface transport. The model uses Monte Carlo simulations to calculate the risk of infection, based on the distribution of the input data, as well pathogen-specific dose response parameters (Schijven et al., 2011). This is done in order to reach a health based target which is the criterion to minimize the risk of infection below 10^{-4}/person/year, based on recommendations by the WHO (World Health Organization, 2017a). To reach this target, the input parameter of total subsurface removal in QMRAspot was adjusted so that the 95th-percentile of the estimated risk was below 10^{-4}/person/year. The input parameters for the program are given in Table 3.2. The source concentrations of *Cryptosporidium* and *Campylobacter* were obtained from Demeter et al. (2021), who used a probabilistic-deterministic water quality model of the Danube, which considered the major wastewater sources upstream of the study site. This water quality model produced a mean and 95th percentile concentration for each pathogen, from which QMRAspot computed a concentration distribution to run 10.000 Monte Carlo simulations, resulting in a specific risk (infections/person/year). Two separate inactivation rate coefficients were used for *Campylobacter*, because of large differences in the literature. For *Cryptosporidium*, these differences were smaller and therefore of less influence on the resulting setback distances, which is why only one value was used. Dose-response curves for each pathogen are included in the program (Schijven et al., 2011; Teunis et al., 2005, 2002).

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Table 3.2. Pathogen-specific input parameters used for QMRAspot and calculation of setback distances

Pathogen	Parameter	Unit	Value	Reference
Cryptosporidium	River conc. (mean, 95th %)	n/l	0.15, 0.8	(Demeter et al., 2021)
	First order inactivation	day^{-1}	0.011	(Sidhu et al., 2010)
Campylobacter	River conc. (mean, 95th %)	n/l	0.8, 4.64	(Demeter et al., 2021)
	First order inactivation	day^{-1}	5	(Sidhu et al., 2010)
			0.11	(Ross et al. 2006)

3.2.5 Analytical methods

BTCs for the conservative tracers and *B. subtilis* were plotted as sample concentrations over time, normalized by the initial concentration (in C/C_0). From this data, spatial removal rates λ (1/L) for *B. subtilis* spores were calculated for each test as per Equation 3.5, which is valid for three dimensions if the flow rate is constant (Kretzschmar et al., 1997; Pang, 2009):

$$\lambda = -\frac{ln\left(\frac{Q}{N_0}\int_0^{t_f} C(t)dt\right)}{x} = -2.3\frac{log_{10}\left(\frac{Q}{N_0}\int_0^{t_f} C(t)dt\right)}{x} \qquad (3.5)$$

where Q is the flow rate in the well (L^3/T), N_0 is the total amount of microbial tracer injected (M), C(t) the concentration at a given time, t, after injection (M/L^3), t_f is the final time of the test (T), and x the distance travelled to the pumping well (L). The integration was approximated by dividing the time series into sampling intervals, of which it was assumed that the sample concentration was an average value for that time interval.

The setback distance for the QMRA method was calculated by the 1D advection-dispersion equation coupled with the removal, λ, and inactivation rate (modified from Blaschke et al. 2016):

$$x = 2.3\frac{2\alpha_l\, log(F)}{1-\sqrt{1+4\alpha_l\left(\frac{\lambda\cdot v+\mu}{v}\right)}} \qquad (3.6)$$

where F is calculated by QMRAspot and is the required removal of *Cryptosporidium* or *Campylobacter* by subsurface transport (as a fraction) to meet the health based target, α_l is the longitudinal dispersivity (L), and μ is the inactivation rate (1/T). Here, the values of λ found by the microbial tracer tests were used, as well as values for the estimated upscaled-λ

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(see Chapter 3.2.6). As an additional comparison, a high and low value were used, taken from the range of λ for sand and gravel in the literature (Pang, 2009).

As a comparison to the model-derived 60-day TOT described above, the setback distance based on the Austrian 60-day TOT regulation was calculated using Equation 7 (ÖVGW, 2004):

$$t = \frac{0.462 \cdot 0.045 K_f}{U} \cdot \left[x - \frac{Q}{U \cdot h_{GW} \cdot 2\pi} \cdot ln\left(1 + \frac{x \cdot U \cdot h_{GW} \cdot 2\pi}{Q}\right)\right] \qquad (3.7)$$

where K_f is the hydraulic conductivity (L/T), U is the Darcy velocity (L/T) and h_{GW} is the height of the water column in the aquifer (L).

3.2.6 Upscaling of λ

In this study, the values for λ were found for a distance of 25 m and may not be representative for larger transport distances, as λ has been reported to decrease with an increase in travel distance (Hornstra et al., 2018; Kvitsand et al., 2015; Schijven and Šimůnek, 2002). Explanations for this phenomenon include distance-related straining processes, reduction of the ionic strength of the input solution over time due to dilution, blocking of favourable attachment sites, increasingly anoxic conditions away from the injection well, and bacterial heterogeneity (Bradford et al. 2007; Pang 2009; Camesano et al. 1999). Because the required setback distances are likely an order of magnitude larger than this distance, the obtained values for λ from the experiments may yield setback distances that are too short, which could lead too high risk levels. To solve this problem, an upscaled λ was estimated using the literature comparison in Oudega et al. (2021), from which a relationship between travel distance and λ can be formulated (Figure 3.2). The relationship of y (corresponding to $\lambda_{field}/\lambda_{column}$) and x (corresponding to transport distance) was then used to calculate the ratio of $\lambda_{field}/\lambda_{column}$ at larger transport distances. Because the value of λ_{column} is known, λ_{field} can be calculated at a different transport distance. Because the relationship as found in Fig. 2 has no data points with a transport distance larger than 97 m (Harvey et al., 2008), scaling beyond this point would be unreliable. Therefore, a transport distance of 96 m was chosen as a characteristic length for upscaling λ.

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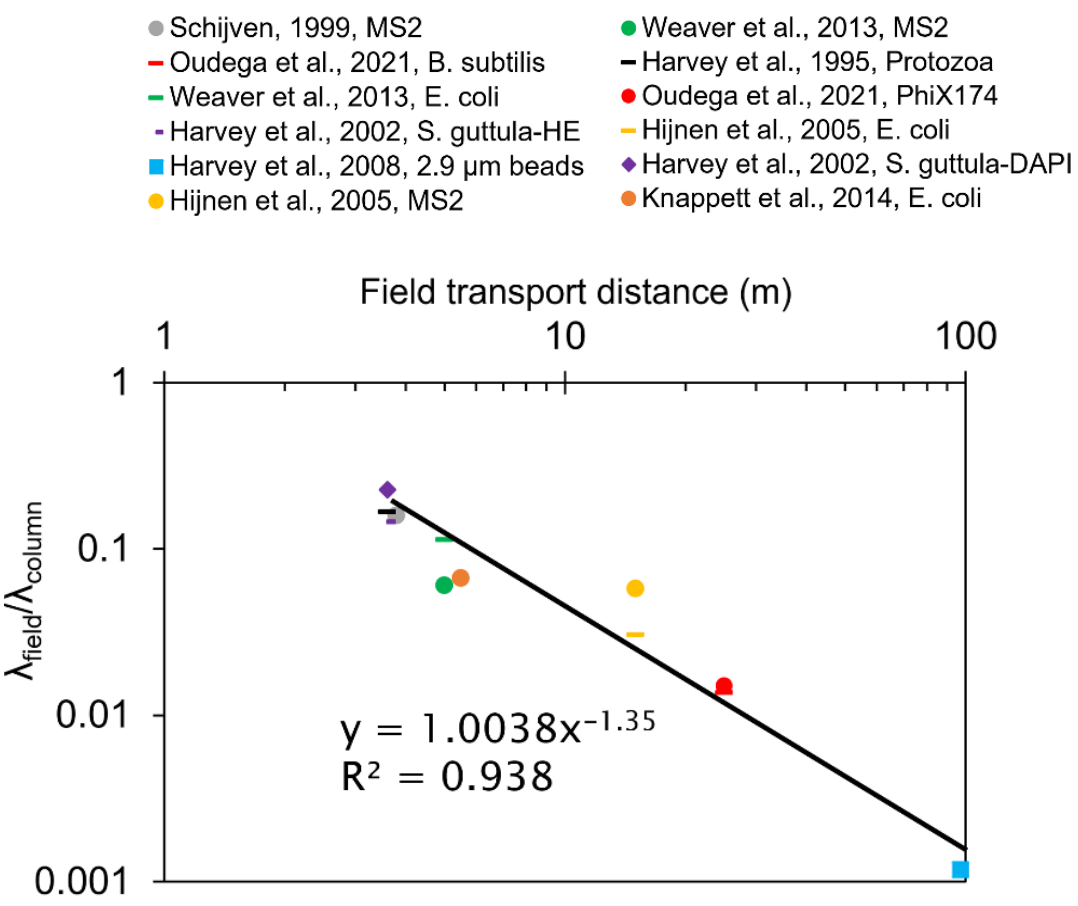

Figure 3.2. Comparison of the ratio of $\lambda_{field}/\lambda_{column}$ between studies with different transport distances. Modified from Oudega et al. (2021).

Since this relationship is merely a first step to understanding the upscaling relationship at different transport distances, using location-specific data, the resulting λ values have a very high uncertainty. Therefore, the upscaling of λ is used here only to showcase how setback distances could change if tracer tests are performed at different distances, highlighting the importance of reliably determining λ.

3.3 Results

3.3.1 Comparison of bromide and uranine as conservative tracers

To ascertain that direct comparisons were possible between bromide and uranine, a test with both conservative tracers was carried out. The results show that bromide and uranine behave similarly in terms of peak timing and change in concentration (Figure 3.3). Note that bromide was injected in a 100 times larger volume than uranine, hence the 2-log difference

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in C/C_0. This may also change flow properties (such as dispersion) between the tests, leading to slightly differing results. A further explanation of the differences between the two conservative tracers is that uranine may experience sorption to aquifer grains during subsurface flow (Dollinger et al. 2017). As bromide does not experience sorption, a higher concentration may be transported to the pumping well. Lastly, the difference in sampling and measurement methods may cause differences between the BTCs: bromide was sampled by an autosampler, while uranine was measured in the flow-through cell with a fluorescence meter, which is an advantage over sampling because of the high temporal resolution. The recoveries were ~67.7% for bromide and ~63.3% for uranine. This underrecovery is likely due to trapping of these tracers in immobile pore water, possibly due to matrix diffusion (Boggs and Adams 1992), although the spatial variability in hydraulic conductivity has also been presented as a reason for this (Fiori et al. 2019). The tailing in both tracer's BTCs shows that the pore velocity was not constant for the entire tracer plume, which can also be explained by heterogeneity in hydraulic conductivity in the soil (Fiori et al. 2019), as well as flow accelerating towards the well screen due to extraction of water. The similarities between the breakthrough of the two tracers were considered sufficient to approve using uranine at this study site as a conservative tracer.

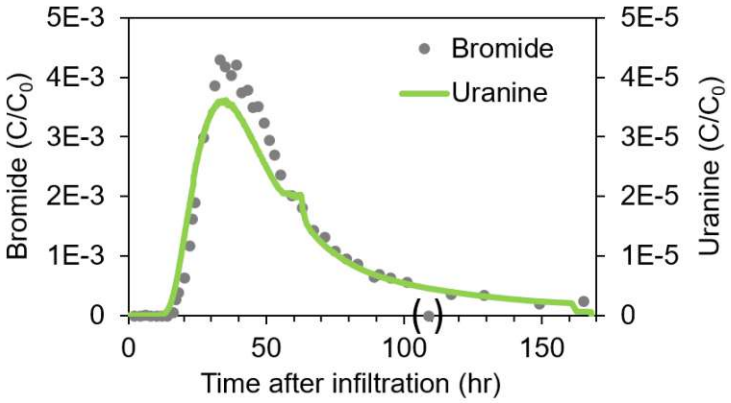

Figure 3.3. Comparison of conservative tracers. Outliers between brackets.

3.3.2 Bacillus subtilis spores breakthrough and model results

In tests 1a-b, the *B. subtilis* concentrations are in the range of the limit of quantification due to the low concentrations in the samples (Fig. 3.4-1a and 1b). At t≈30 h, *B. subtilis* peaked earlier than the conservative tracer, showing that pore-size exclusion is an important process in the study area (Grindrod et al., 1996; Pang et al., 2005). This, in turn, affects the

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dispersivity values in the model, which were lower for *B. subtilis* than for the conservative tracers (Table 3.3). This is because the microbial tracers travel predominantly through larger and better-connected pores, leading to a more advective and less dispersive transport than the conservative tracers (Pang et al., 1998). The peak C/C_0 breakthrough was $4.2 \bullet 10^{-9}$, showing a peak reduction of just under 9-log. The very early breakthrough in test 1a was assumed to be sample contamination. Both tests exhibit significant tailing, as well as breakthrough of higher concentrations after the initial peak has passed (the high concentration in spore samples at t=60 h in both tests 1a and 1b).

In tests 5a-b, peak concentrations were around 4 times higher than in tests 1a-b. Furthermore, peak time was much earlier than the conservative tracer peak. The reduction in peak concentration was approximately 8-logs. The outliers were likely a product of sample contamination.

In tests 10a-b, BTCs were less irregular than in the lower pumping rate tests, which can be attributed to the higher concentrations in the analyzed samples. Concentrations peaked at approximately t=4.69 h with C/C_0 at 3.75×10^{-8}, around 3-4 times higher than tests 5a-b. An overview of the tests is shown in Table 3, with the removal rates for *B. subtilis* as calculated per Equation 5, and values for K_{att} and K_{det} taken from the modeling study.

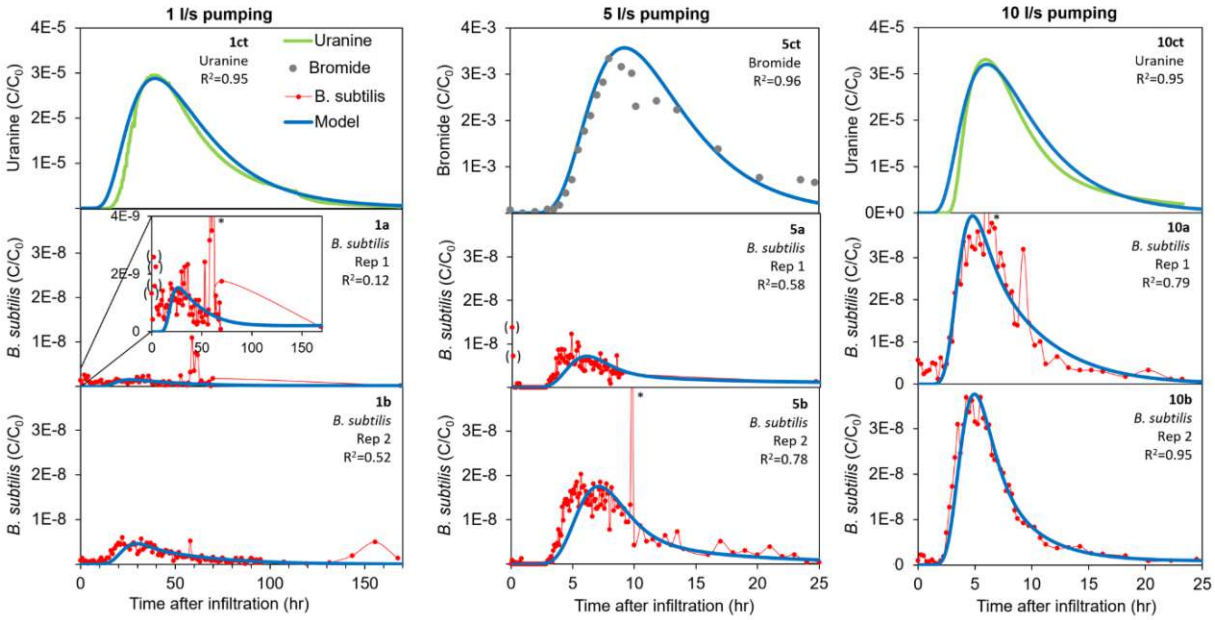

Figure 3.4. The BTCs of the tracer tests and modeling study, where *ct* stands for conservative tracer test, and *a* and *b* are duplicates of the microbial tracer test. Asterisks (*) stand for outliers with concentrations above the maximum y-axis value. Figures *5ct*, *a* and *b* modified from Oudega et al. (2021).

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Table 3.3. Results of field tests and modeling study.

	Test	1 Pre	1a	1b	5a[a]	5b[a]	10a	10b
	Pumping rate (l/s)	1.1	1.2	1.0	5.1	4.7	9.6	9.5
	Average flow rate (m/d)	2.4	2.4	1.9	15	13	24	23
	Gradient P24 – LB13 (‰)	7.5	5.3	5.9	13	13	25	25
Bromide	Peak concentration (C_{max}/C_0)	4.3×10^{-3}	-	-	3.3×10^{-3}	7.4×10^{-3}	-	-
	Recovery (%)	68	-	-	71	93	-	-
	Time to peak (h)	33	-	-	8.0	10	-	-
	Longitudinal dispersivity (m)	1.8	-	-	1.8	1.8	-	-
	Transverse dispersivity (m)	0.18	-	-	0.18	0.18	-	-
Uranine	Peak concentration (C_{max}/C_0)	3.6×10^{-5}	2.7×10^{-5}	2.9×10^{-5}	-	-	3.1×10^{-5}	2.9×10^{-5}
	Recovery (%)	63	58	51	-	-	75	84
	Time to peak (h)	36	48	39.	-	-	5.8	6.1
	Longitudinal dispersivity (m)	2.9	2.9	2.9	-	-	2.9	2.9
	Transverse dispersivity (m)	1.1	1.1	1.1	-	-	1.1	1.1
B. subtilis	Peak concentration (C_{max}/C_0)	-	2.3×10^{-9}	6.0×10^{-9}	1.1×10^{-8}	2.0×10^{-8}	3.8×10^{-8}	3.7×10^{-8}
	Recovery (M_{out}/M_{in})	-	7.1×10^{-4}	6.9×10^{-4}	1.1×10^{-3}	1.8×10^{-3}	9.3×10^{-3}	7.5×10^{-3}
	Time to peak (h)	-	36	22	4.8	5.7	6.5	4.3
	Longitudinal dispersivity (m)	-	1.2	1.2	1.2	1.2	1.2	1.2
	Transverse dispersivity (m)	-	0.15	0.15	0.15	0.15	0.15	0.15
	λ (log/m)	-	0.28	0.27	0.27	0.26	0.19	0.20
	K_{att1} (hr^{-1})	-	0.18	0.12	0.76	0.60	0.53	0.66
	K_{det1} (hr^{-1})	-	0.00080	0	0.0040	0.0008	0.00010	0.0013
	K_{att2} (hr^{-1})	-	0.12	0.090	0.20	0.12	0.42	0.22
	K_{det2} (hr^{-1})	-	0.10	0.030	0.21	0.20	0.37	0.40

[a] Values taken from Oudega et al. (2021), of which some were altered due to re-measurement of piezometer coordinates at the study site and subsequent changing of the transport distance.

3.4 Discussion

3.4.1 HYDRUS-3D Modeling

The ratio of C_{max}/C_0 for *B. subtilis* spores in the tracer tests was generally 10^{-8}–10^{-9} compared to 10^{-3} for the conservative tracers (Table 3.3). This shows that removal processes are important for colloid transport in this area. Many studies have ascertained that no significant inactivation of *B. subtilis* spores took place after time periods from 7 days up to 45 days (Greskowiak et al. 2006; Li et al. 2018; Pang et al. 1998; Pike et al. 1969; Ratcliffe 1995), and because the fraction of colloid size to median grain size (0.0003 in our experiments) is

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smaller than 0.0017, straining should not occur (Bradford et al., 2002b). For these reasons, the removal of *B. subtilis* in this study was assumed to be governed by attachment.

Two attachment and detachments rate coefficients were necessary to accurately model the tailing of the *B. subtilis* BTCs. In this model, K_{att1} mainly controls the height of the peak concentration, while K_{att2}, K_{det1} and K_{det2} mainly control the shape of the falling limb of the BTC (Schijven et al. 2001). Table 3 shows the attachment and detachment rate coefficients that were found for each experiment. The difference between attachment sites can be explained by microbial population heterogeneity; i.e. certain individual organisms within the colloidal population are inherently more or less likely to attach to sorption sites due to differences in their surface structure or charge (Foppen and Schijven, 2006; Schijven and Hassanizadeh, 2000). However, another explanation is that physical heterogeneity is more important; i.e. certain attachment sites are more likely to capture *B. subtilis* spores than others. This could depend on multiple factors, for example the extent of the favorable attachment sites, or their charge (i.e. iron content) (Ginn et al., 2002; Knapp et al., 1998; Schijven and Šimůnek, 2002).

It was found that K_{att1} was lowest for the lowest flow rate tests (1 l/s), but in the middle and highest flow rate tests (5 and 10 l/s respectively), K_{att1} was similar. Other studies have also found an increase in attachment rate with pore-water velocity (v) on both the column and field scale (Hendry et al. 1999; Hijnen et al. 2005; Schijven et al. 2001). K_{det1}, K_{att2} and K_{det2} were also found to increase with v. This shows that the ratio of reversibly attached particles to irreversibly attached particles increases with v, which affirms previous findings (Bradford et al., 2016b). An increase in reversible attachment might be an important mechanism that causes removal to decrease with an increase in flow rate.

Even though the attachment rate coefficients increased with flow rate, overall removal decreased due to shorter residence times; a pumping rate change from 1 to 10 l/s decreased the removal rate (λ) by more than 30% (Table 3.3). This is supported by the literature on field tracer studies, which generally states that an increase in flow rate decreases removal (Camesano and Logan, 1998; Kvitsand et al., 2015; Pang, 2009), which in this study ranges from 0.19-0.28 log/m. Typically, values for λ are in the range of 10^{-2}-10^{-1} log/m for sand and gravel aquifers, but in gravel aquifers with a flow rate >11 m/d, can be as low as 10^{-3} log/m (Pang, 2009). The values for λ in this study are on the higher side of this range, which might

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be due to the high amount of fine material present at this study's field site; the D_{50} is 4 mm versus, for example, 18 mm at the Burnham test site (Pang et al., 2005). Another explanation is that there could be differences in sphericity and/or surface roughness of the material, which can influence attachment (Saiers and Ryan, 2005; Shellenberger and Logan, 2002). Lastly, the high iron content in the groundwater of our study site points to the presence of iron-oxides, which can function as attachment sites, especially close to the injection well where at least some amount of oxygen was introduced, leading to enhanced microbial attachment (Ginn et al. 2002; Knapp al. 1998).

3.4.2 Setback Distances

The computations with QMRAspot showed that in order to keep the 95th percentile of the risk of infection below 10^{-4}/person/year, the required total subsurface removal was 5.6 log for *Cryptosporidium* and 6.8 log for *Campylobacter*.

Table 3.4 shows setback distances calculated for each pumping rate, based on the 60-day TOT (as per Equation 3.7, as well as extrapolated from model results), and based on the QMRA (Equation 3.6) for each pathogen. The results show that a pumping rate increase from 1 to 10 l/s can lead to a 1.0-3.8 times greater setback distance, depending on the method used. Setback distances for *Campylobacter* were similar or smaller than for *Cryptosporidium* due to the higher inactivation rates (0.11-5 versus 0.011 log/day, respectively) (Sidhu et al., 2010). However, *Cryptosporidium* is larger than *B. subtilis* and might therefore experience additional removal processes such as straining and/or wedging (Headd and Bradford, 2016; Li et al., 2006; Tufenkji et al., 2004). Therefore, the setback distances for *Cryptosporidium* can be regarded as conservative. For low values of λ, setback distances for *Campylobacter* varied more with pumping rate than for higher λ. This is because at lower λ, inactivation becomes more important due to the longer travel times.

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Table 3.4. Calculated setback distances based on a 60-day TOT, as well as setback distances based on the QMRAspot for different values of λ

Test	1a	1b	5a	5b	10a	10b
Experimental λ (log/m)	0.284	0.274	0.273[a]	0.257[a]	0.189	0.198
Estimated λ[b] (log/m)	0.028	0.027	0.027	0.025	0.018	0.019
Setback distances (m) based on 60-day TOT						
Model-derived[b]	477	523	585	524	736	657
Austrian regulation[d]	176	174	372	322	493	473
Setback distances (m) based on required reductions estimated by using QMRAspot						
Cryptosporidium spp. (μ = 0.011 log/d)						
Using experimental λ	57	59	59	62	81	78
Using estimated λ	471	487	496	525	709	678
Using λ = 10^{-1} log/m	142	142	143	143	143	143
Using λ = 10^{-3} log/m	7752	7723	8153	8036	8394	8860
Campylobacter spp. (μ = 5-0.11 log/d)						
Using experimental λ	41-69	39-70	62-72	63-76	84-98	81-94
Using estimated λ	65-479	58-488	168-556	160-576	231-785	221-754
Using λ = 10^{-1} log/m	55-164	50-163	109-171	106-170	124-172	123-172
Using λ = 10^{-3} log/m	70-2146	62-2111	206-2819	190-2634	278-3176	266-3686

[a] Values taken from Oudega et al. (2021), [b] The *estimated* (or upscaled) *λ* is the value acquired by the upscaling procedure based on Figure 3.2, while the *experimental λ* is the value measured in this study, [c] As calculated from the modeled groundwater gradient, [d] As per Equation 7.

The setback distances for both pathogens, as calculated with the estimated upscaled-λ (~700m for the highest pumping rate), are similar to the model-derived 60-day TOT setback distances. This shows that, at least in this study, the best estimates for setback distances based on QMRA do not differ greatly from the 60-day TOT distance, and highlights that the TOT method is still viable if the necessary data for QMRA is not available, especially when considering its greater ease of use. However, whereas the 60-day TOT method is considered to be sufficient for all pathogens, the QMRA in this study was only done for *Cryptosporidium* and *Campylobacter*. Other pathogens might have different source concentrations, removal parameters and dose-response curves, which could lead to vastly different setback distances. Still, these results are in contrast with findings by Schijven et al. (2006), who calculated by QMRA that protection zones of 1-2 years were needed to not exceed the same risk level. However, Schijven et al. (2006) did their analysis for viruses in raw sewage water

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leaking through a pipe into a uniform sand aquifer. The situation in this study is very different, as it assumes infiltration of river water through the riverbank. Even though pathogen concentrations in a river can increase drastically after heavy rainfalls, strongly affecting setback distances, this study accounts for this variability in source concentration by using results from a probabilistic-deterministic water quality model (Demeter et al., 2021).

Important parameters in the calculation of setback distances by QMRA are λ and inactivation rate (μ). Because it is not easy to find accurate values for these parameters, literature values are often used instead. A challenge is that a large range of values exists in the literature, even for the same aquifer type as is the case for λ in gravel aquifers (Pang, 2009). This is shown in Table 3.4, where using different λ for alluvial gravel aquifers can yield vastly different setback distances. In the case of *Campylobacter*, setback distances varied by 1-2 log, depending on which value of μ was chosen, which shows that the effect of μ on setback distance is greater than that of pumping rate (or flow gradient). This underlines the importance of obtaining reliable μ and λ values for the calculation of setback distances with QMRA. In-situ batch tests and field tracer tests are the most reliable methods to determine μ; however, besides the practical difficulties inherent in carrying out these tests, interpreting the resulting λ values poses further difficulties because λ can change with pumping rate as well as with scale. Therefore, it is additionally important to consider scaling for the calculation of setback distances with QMRA. Unfortunately, our methods of upscaling are not yet adequate for reliably estimating λ at larger scales. Ideally, there would be multiple sampling points to capture the effects of changing λ with distance, or microbial tracer tests would be done from the furthest possible point from the drinking water well, such as a river.

To be able to accurately calculate setback distances based on QMRA, it is important not only to obtain reliable transport parameters, but also to understand the hydrogeological setting. If the source of pathogens is a river, there might be additional inactivation due to sunlight exposure (Bambic et al., 2015; Pitkänen and Hänninen, 2016). If the river has a static riverbank, a colmation layer may be present at the river-aquifer interface, which would dramatically increase removal (Derx et al. 2014; Derx et al. 2010). On the other hand, if the riverbank is natural, or if the likely source of pathogens is from a pipe located near the saturated zone, this attenuation effect would be largely diminished.

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3.5 Conclusions

This study shows that when removal, λ, is low, varying the flow rate can have a large influence on setback distance calculations. Inactivation, μ, is also an extremely important parameter when the value of λ is small and setback distances are calculated with a QMRA approach. The literature range of λ (and in the case of some pathogens, μ) is very broad, which can lead to inaccurate setback distances. For this reason, tracer tests with surrogates should be performed at the site of interest, but care should be taken as to which surrogates are used, as well as which transport distance should be used to reliably determine λ. Because it is not always possible to carry out tracer tests over large enough distances, it is important to create more reliable upscaling methods, by performing more tracer test studies at different field scales (i.e. field studies in the same aquifer, but with different transport distances), as well as studies at the mesoscale, for example, in large (> 1m) columns.

This chapter was based on the following publication:

Oudega, T.J., Lindner, G., Derx, J., Farnleitner, A.H., Sommer, R., Blaschke, A.P., Stevenson, M.E., 2021. Upscaling Transport of *Bacillus subtilis* Endospores and Coliphage phiX174 in Heterogeneous Porous Media from the Column to the Field Scale. Environ. Sci. Technol. 55, 11060–11069. https://doi.org/10.1021/acs.est.1c01892

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4 Evaluating a robust and easy-to-use biological-activity-based method to assess the presence of potentially adverse bacteria at two riverbank filtration sites along the Danube river: a case study

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Abstract

The Laboratory Biological Activity Reaction Test (LAB-BART) is an easy-to-use assay that utilizes metabolic capabilities to process an array of substrates to semi-quantitatively assess the presence of potentially adverse bacteria in a groundwater sample. Here, we evaluated LAB-BART for the assessment of groundwater samples obtained under real-life conditions from two riverbank filtration (RBF) sites in Austria. Samples were taken monthly for an overall experimental period of six months and analyzed following the manufacturer's recommendations for measuring iron-related, sulfate-reducing, slime-forming and denitrifying bacteria. Additional measurements were done for chemical composition, as well as gene metabarcoding to evaluate the suitability of LAB-BART by identifying relevant bacteria. Results imply that while LAB-BART could not give detailed information on bacterial concentrations, it may be able to indicate hydrological changes in a subsurface system, thus allowing operators to determine an adequate response to a potential influx of undesired bacteria. Despite its limitations, LAB-BART may therefore be a valuable tool for monitoring purposes due to its ease of use, but more research is necessary to determine its accuracy in measuring bacterial activity.

4.1 Introduction

Microbial water quality measurements have been shown to be crucial to ensure consumer safety in the last century (World Health Organization, 2017a). Besides public health benefits, knowledge about the presence or absence of microorganisms associated with non-toxic but undesirable biofouling and microbially induced corrosion allows for timely interventions by the operator of groundwater wells, drinking water production facilities and in the overall drinking water distribution infrastructure (Abdullah et al., 2014). While simple and reliable culture-based assays such as heterotrophic plate counts have long been implemented by policy makers worldwide (e.g., AWWA, DIN TS ISO and AGES, the Austrian Agency for Health, and Food Safety) to ensure biological stability in drinking water, they are known to have certain limitations in indicating viable but non-cultivable (VBNC) bacteria. Advanced assays

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based on the detection of known and unknown genome sequences associated with bacteria of interest such as (quantitative) polymerase chain reaction (PCR) or genome sequencing (e.g., 16S gene metabarcoding) have been proposed to compensate for this, but they are cost- and labor-intensive and therefore seldomly applicable for routine operations. Furthermore, the time between taking the samples and the availability of the result ranges between several days (PCR) and multiple weeks (genome sequencing), which can be problematic for consumer safety. Ready-to-use assays targeting bacterial metabolism such as LAB-BART (Droycon Bioconcepts Inc., Regina, Canada), on the other hand, promise to show results within a few days if bacterial activity is high, and in contrast to classical microbiological methods, LAB-BART can be used at the sampling site without laboratory equipment or trained personnel (Cullimore, 1999).

The main reason to investigate the contamination/colonization of ground water wells for microorganisms is the occurrence of plugging/clogging due to microbial fouling, which in turn can cause production failure and consumer hazards. Next to manganese metabolizing bacteria, this plugging is often caused by the presence or influx of iron-related bacteria (IRB), whose build-up in the well can lead to losses in groundwater flow of as much as 90% (Cullimore & McCann 1977). Microbially induced corrosion, during which microorganisms residing in pipe-adherent biofilms affect their integrity is another common cause of operational concern, which is commonly associated with sulfate-reducing bacteria (SRB) (Cullimore and Johnston, 2004).

Here, we assessed the ready-to-use LAB-BART assay to evaluate the presence of iron-related, sulfate reducing, denitrifying and slime-forming bacteria in two well fields in Austria over the duration of six months under real-life conditions. The results were compared to chemical measurements of the same samples. To assess the suitability of the chosen assays, microbial communities in the samples were further characterized using 16S gene metabarcoding, a sophisticated genome-based method that gives detailed insights into the composition of groundwater microbiomes (Shaw et al., 2015).

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4.2 Methods

4.2.1 Study sites

Samples from two drinking water production sites situated in Lower Austria (Site A and Site B) were utilized to investigate the suitability of the LAB-BART system to identify relevant microorganisms. Both sites use riverbank filtration (RBF) for drinking water production and are equipped with on-site oxygen enrichment.

Site A consists of three sampling points at 8-11 m depth from the ground surface: piezometers A1 and A2, as well as a pumping well: Well A (Fig. 4.1). The groundwater sourced from Well A is continuously fed by water from a nearby, stagnant backwater. After infiltration into the riverbank, the groundwater travels through the gravel aquifer northwards towards Well A. Oxygen enrichment wells are situated northwest of Well A, but because of their location, have little effect on the groundwater in the well. The time between infiltration and pumping is estimated to be 2 months. The operator of Site A reported that Well A is clogged, leading to reduced yield. These issues are associated mainly with manganese but also with iron.

Site B also consists of three groundwater sampling points, one of which is a pumping well. Unlike Site A, successful in-situ oxygen enrichment is employed in between piezometers B1 and B2 to improve water quality, especially to oxidize unwanted metals such as iron and manganese. The groundwater at Site B originates mainly from the Kamp River, a tributary to the Danube River, but also from ambient groundwater sources.

Samples from both sites were taken monthly at the two piezometers and the respective pumping well for a total period of six months in 2019. Samples were immediately stored at 4°C and transported to the laboratory for analysis within 24 hours.

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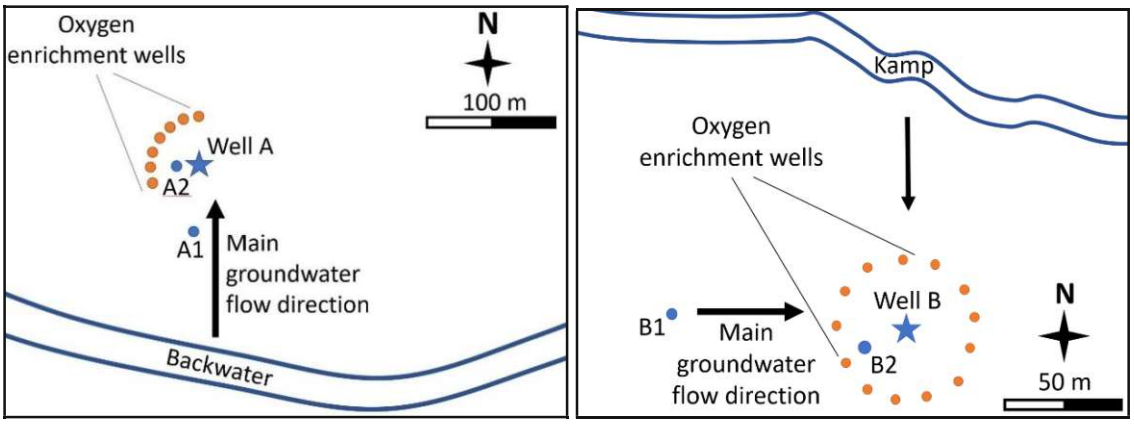

Figure 4.1. Overview of Site A (left) and Site B (right), with oxygen enrichment wells in orange, and sampling wells in blue.

4.2.2 LAB-BART analysis

The LAB-BART test kits consist of tubes containing a pellet of substrate that, in the presence of the defined group of bacteria, is metabolized into a visible color compound (Droycon Bioconcepts Inc. 2004). The approximate concentration as colony forming units per ml of the target bacteria is then determined by the duration until the color change is observable (time lag). Unlike plate-based assays, LAB-BART also accounts for VBNC bacteria and can in theory provide a more realistic estimate of the quantity of viable microbes present (Cullimore 1999). The LAB-BART system is available in nine different kits, each assessing distinct physiological groups of bacteria or algae. Here, we employed the IRB-BART targeting iron-related bacteria, SRB-BART targeting sulfate-reducing bacteria, DN-BART targeting denitrifying bacteria, and SLYM-BART targeting slime-forming bacteria. Detailed information on these bacterial communities, as well as protocols for the specific test kits can be found at the manufacturer's homepage.

For each sample taken at the well fields, 15 ml of groundwater was aseptically transferred into LAB-BART test tubes, which were then kept in the dark and at room temperature. Tubes were observed for a total of 11 to 15 days, and any change in color was documented. In case of the SLYM-BART test kits, additional documentation with fluorescent light was necessary to monitor for the occurrence of fluorescent bacterial metabolites in the tubes. Changes in color and appearance of foam in the tubes corresponded to an approximate bacterial population as colony forming units per milliliter (cfu/ml) according to Table 4.1.

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Unfortunately, no error is given for these “approximate concentrations” in the LAB-BART protocol (Droycon Bioconcepts Inc. 2004), and the qualitative assessment of “aggressive”, “moderate” or “not aggressive” are not defined, besides the fact that aggressive bacteria mean active bacteria.

Table 4.1. The corresponding concentrations (cfu/ml) for the day of color-change (time lag) for each test kit, according to the LAB-BART protocol. Between brackets is the qualitative assessment of the sample, where A. = Aggressive, M. = Moderate, N.A. = Not Aggressive.

Days	IRB	SRB	DN	SLYM
1	570,000 (A.)	2,200,000 (A.)	1,800,000 (A.)	1,750,000 (A.)
2	140,000 (A.)	500,000 (A.)	215,000 (A.)	440,000 (A.)
3	35,000 (A.)	115,000 (A.)	25,000 (M.)	67,000 (A.)
4	9000 (A.)	27,000 (A.)	3000 (M.)	13,000 (M.)
5	2200 (M.)	6000 (A.)	350 (N.A.)	2500 (M.)
6	500 (M.)	1400 (M.)	<50 (N.A.)	500 (M.)
7	150 (M.)	325 (M.)	<50 (N.A.)	100 (N.A.)
8	25 (M.)	75 (M.)	<50 (N.A.)	<20 (N.A.)
9	8 (N.A.)	20 (N.A.)	<50 (N.A.)	<20 (N.A.)
10	<1 (N.A.)	5 (N.A.)	<50 (N.A.)	<20 (N.A.)
11	<1 (N.A.)	<1 (N.A.)	<50 (N.A.)	<20 (N.A.)

4.2.3 Chemical analysis

In addition to the LAB-BART test, 35 chemical elements were determined in all samples by high resolution inductively coupled (sector field) mass spectrometry (HR-ICP-MS) with a Finnigan Element 2 mass spectrometer (Thermo Fisher Scientific, Germany) at the IFA-Tulln. All unfiltered samples were acidified with ca. 1% ultrapure HNO3 (ROTIPURAN Supra, 69%, Carl Roth, Karlsruhe, Germany) to reach a pH of 1-2 in order to avoid precipitation and minimize biological activity within 24 hours of sampling. Scandium, indium, and thallium were added as internal standards for quality control, in concentrations of 20 µg/l, 10 µg/l, and 10 µg/l, respectively. Temperature and dissolved oxygen were measured at the sampling sites, while other basic parameters such as pH, electrical conductivity (EC), chloride, nitrate, nitrite, sulfate, phosphate, iron and dissolved organic carbon (DOC) were determined in a commercial ISO 17043 accredited laboratory. Parameters unlikely to directly influence the LAB-BART results are not shown in this publication.

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Samples with a high amount of iron related bacteria (IRB) were expected to contain either high concentrations of insoluble ferric forms of iron (e.g., Fe(OH)3) or soluble ferrous forms of iron (e.g., Fe2+), as these bacteria can either reduce or oxidize iron in groundwater. High amounts of sulfate reducing bacteria (SRB) were expected to lead to the presence of hydrogen sulfide (H2S) under anoxic conditions. SRB use hydrogen rather than oxygen as the primary energy source for their metabolism, and should be inhibited by the presence of oxygen (Cord-Ruwisch et al. 1987, Krekeler et al. 1998). Their primary source of sulfur may not be only sulfate, as samples containing organic acids, alcohols and proteins could also lead to the presence of sulfides. Hence, the absence of sulfate does not necessarily mean that SRB should be absent (Plugge et al. 2011). Slime-forming bacteria (SLYM) are able to function in a large range of hydrochemical conditions but form the thickest slimes in oxygen-rich environments. Consequently, a correlation between SLYM activity and oxygen concentrations was expected. Denitrifying bacteria (DN) were expected to be present at locations with high concentrations of nitrate in the subsurface, which they utilize. However, this activity is limited to anoxic conditions in reductive environments.

4.2.4 Microbial community analysis using gene metabarcoding

To analyze microbial communities in the groundwater samples, gene metabarcoding via 16S rRNA gene amplicon sequencing was conducted. For this, approximately 2 l of groundwater was filtered through 0.22 µm polycarbonate membrane filters (Merck Millipore, Germany). Then, DNA extraction was performed using a bead-beating and phenol/chloroform protocol as described elsewhere (Griffiths et al., 2000; Mayer et al., 2018; Reischer et al., 2006). Microbial community composition was determined targeting the highly conserved V3-V4 genome region present in all bacteria (Ong et al., 2013). After amplification using polymerase chain reaction and thorough quality control, the Illumina MiSeq platform (Illumina, USA) was used in accordance with the manufacturer's recommendation. Sequence data was processed in R (R Core Team, USA), using the DADA2 pipeline as described in detail by Callahan et al. (2016).

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4.3 Results and Discussion

4.3.1 Results of LAB-BART analysis

The results of one of the sampling campaigns are shown in Fig. 4.2.

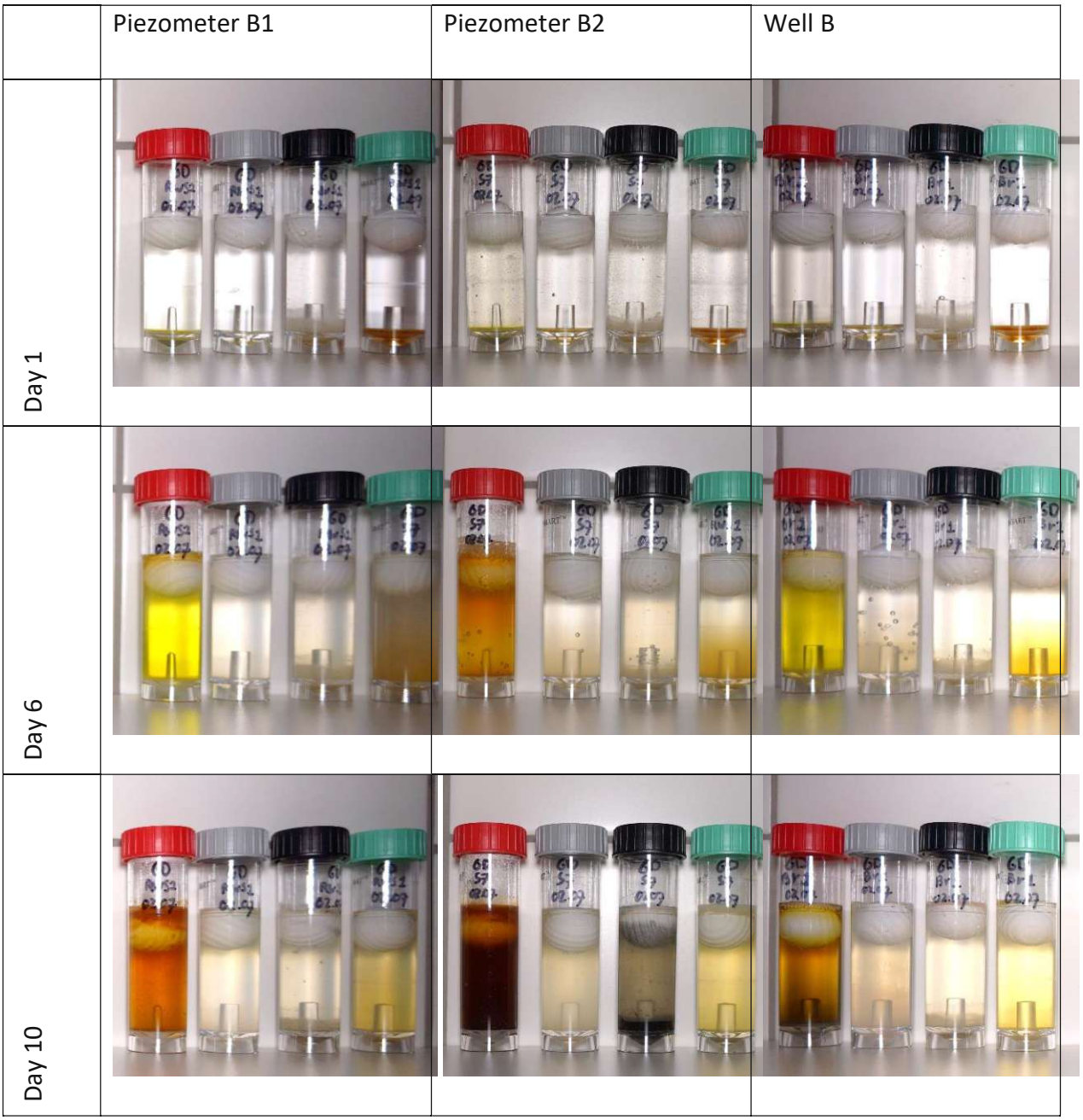

Figure 4.2. Photos of the analysis of the 02.07.2019 sampling campaign at Site B. Vials with red caps are IRB-BART, with grey caps DN-BART, with black caps SRB-BART and with green caps SLYM-BART.

Results of the LAB-BART tests showed that Site A and Site B have some distinct differences with regards to all the utilized test kits (Fig. 4.3).

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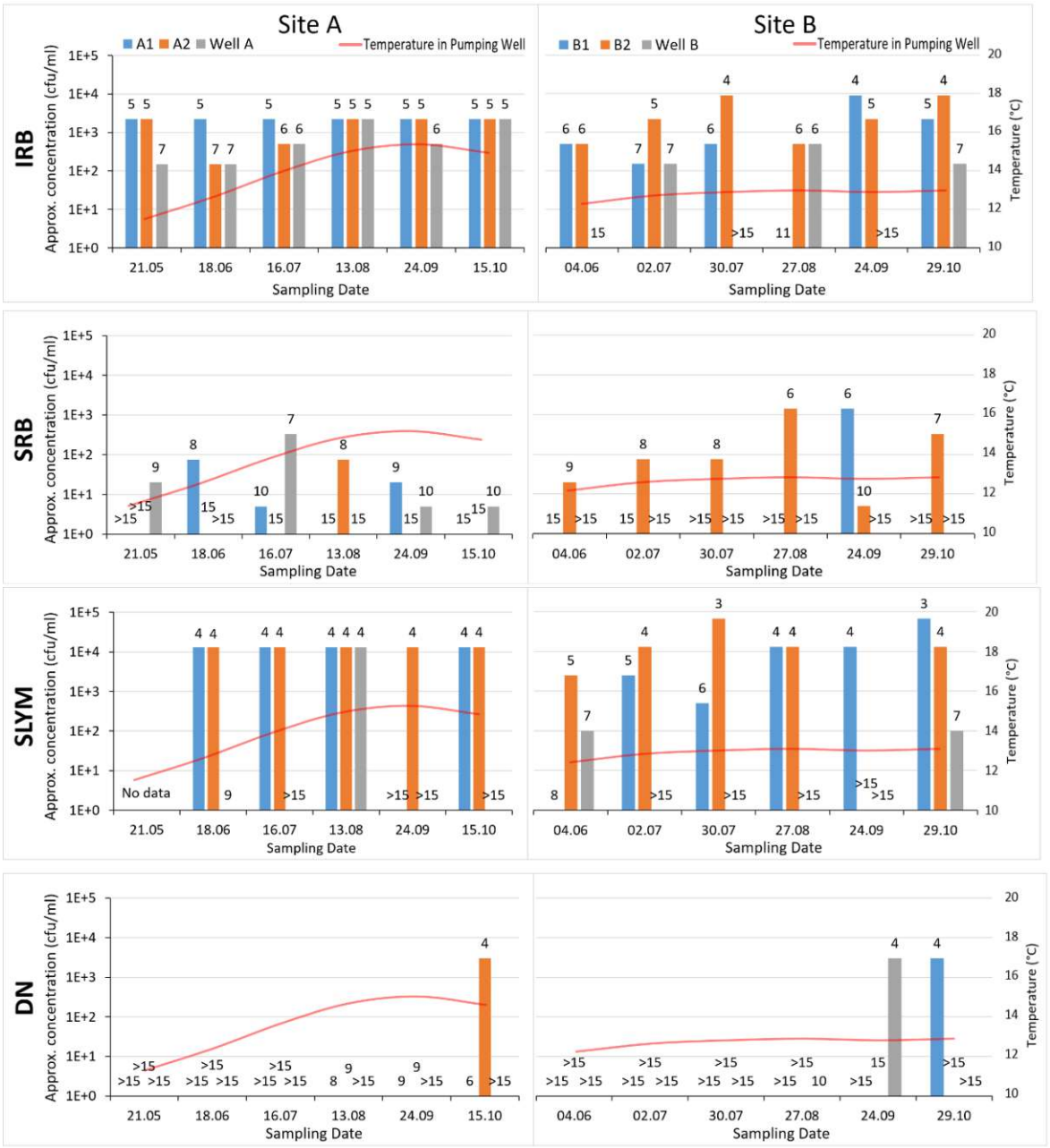

Figure 4.3. LAB-BART results. The number above the bars signifies the number of days before the color change took place (time lag). *Note: the approximate concentration can be zero even if a color change does happen, because it has been too many days (see Table 4.1).*

In Well B, IRB were measured in lower approximate concentration (AC) than in Well A on most of the sampling dates. IRB further seemed to be stable over time as well as distance, especially at Site A. At Site B, the AC of these bacteria was reduced towards Well B, which was not the case at Site A. An explanation for this may be that the oxygenation at Site B reduced these bacteria in the groundwater.

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Results of SRB at Site B showed a persistent increase in AC between piezometers B1 and B2. Because SRB is generally associated with low oxygen environments, it is unlikely that the oxygenation happening between these wells was the cause. However, groundwater samples from B2 were consistently turbid, implying the presence of suspended solids, which may affect the chemical and bacterial composition at that location. In Well B, SRB was not found in any of the samples. This was similar to the IRB results, where ACs in Well B were also generally lower than in B1 and B2.

While SLYM was present in samples from piezometers at both sites, it was barely shown in Well A and B. One explanation for this may be that slime-forming bacteria are more active in slower-flowing waters, and towards the well the groundwater flow rate increases drastically. The mechanical strain from fast-flowing groundwater in the relatively conductive gravel aquifers can inhibit the formation of slimes (Stoodley et al., 1999; Tsai, 2005). At Site B, ACs in B2 were usually the highest (with the notable exception of 24.09.2023, when the AC was zero), which could be an effect of the oxygenation just before this sampling location.

Analysis of the DN test kits mostly resulted in an AC of <50 cfu/ml, the lowest value that these tests can determine (Table 4.1), implying that at these study sites, DN bacteria were of too low concentration to be accurately assessed. While most samples did not show presence of DN, the few samples that did were all taken in late summer or autumn, possibly signifying a temperature dependency.

The results at Site A showed fewer fluctuations over time than at Site B. Firstly, this might be due the source of the water; at Site A it comes from a nearby backwater, while at Site B the groundwater infiltrates both from the Kamp River, a more dynamic waterway, as well as from ambient groundwater flows (Fig. 4.1). Secondly, oxygen enrichment was more successful at Site B, which might affect microbial communities. Lastly, no temporal or seasonal trends could be discovered, implying that the groundwater temperature did not play a significant role in these analyses. However, the sampling period was short (May to October), and therefore these trends may have been missed.

Analysis of Variance (ANOVA) was used to carry out a statistical comparison between the two sites. Assuming a p-value of 0.05, the results showed that the mean ACs at Site A and B were the same. However, as the variance was larger than the mean values, and because of

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the small sample size and semi-quantitative nature of the LAB-BART system, this result may not be meaningful.

4.3.2 Results of chemical analysis

Chemical analysis showed that concentration fluctuations were generally low, and no real temporal trends could be seen in river water nor groundwater, which was similar to the LAB-BART results (Table 4.2). Notable exceptions are the temperature at Site A, which fluctuated by a maximum of 7 °C over the study period, while at Site B the temperature was more stable. This did not seem to influence LAB-BART results. Surface waters at both sites showed an increase in EC with increasing temperatures, but this was not reflected in the groundwater EC, and therefore the effect on LAB-BART results would be minimal. At Site A, oxygenation had only a limited effect on the groundwater, as shown by the fact that oxygen concentrations remained stable throughout the transect, while at Site B, oxygenation did increase the oxygen concentrations between B1 and B2, after which concentrations decreased again towards Well B.

Generally, microorganisms need phosphorus to thrive (Bünemann et al., 2008; Widdig et al., 2019). At both sites, there was abundant phosphorus for microbiological activity in the surface water (~20 µg/l to 130 µg/l), which decreased slightly after infiltration into the subsurface. This could be an indication of bacterial metabolism during subsurface flow, showing that LAB-BART should detect some activity. Both phosphorus and nitrite concentrations showed high temporal fluctuations in the Kamp River at Site B. Interestingly, phosphorus stabilized after infiltration (except for the 04/06 B2 sample, possibly due to the same reason as the iron concentration increase, see below), while the nitrite fluctuations increased in B1. Nitrite concentrations only stabilized after the oxygenation between B1 and B2 and were notably (approx. 2 logs) reduced. At Site A, these fluctuations were much lower, possibly due to the stagnant surface waters at that site. The other parameters were more stable at both sites, besides some smaller (<1 log) fluctuations.

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Table 4.2. Results of the chemical analysis of the groundwater and corresponding surface waters

Site A

	Parameter	Unit	21/05/19	18/06/19	16/07/19	13/08/19	24/09/19	15/10/19	Average
Back-water	Temp.	°C	-	-	-	-	-	-	-
	pH		-	-	-	-	-	-	7.8[a]
	EC	µS/cm	291	295	350	344	353	466	**350**
	DOC	mg/l C	-	-	-	3.0	3.0	2.4	**2.8**
	Oxygen	mg/l	-	-	-	-	-	-	-
	Iron	µg/l	133	112	79	91	83	243	**123**
	Chloride	mg/l	18	13	19	19	18	21	**18**
	Phosphorus	µg/l	19	31	26	33	22	45	**29**
	Nitrate	mg/l	12.0	6.2	3.6	2.9	5.0	2.6	**5.4**
	Nitrite	mg/l	0.074	0.110	0.110	0.036	0.012	0.020	**0.060**
	Sulfate	mg/l	21	17	21	21	22	22	**21**
A1	Temp.	°C	11.4	12.9	15.1	16.7	17.3	14.8	**14.7**
	pH	-	-	-	-	-	-	-	**7.7**
	EC	µS/cm	472	436	450	463	443	449	**452**
	DOC	mg/l C	-	-	-	1.5	2.0	1.2	**1.6**
	Oxygen	mg/l	2.50	0.77	0.91	0.80	0.06	0.27	**0.89**
	Iron	µg/l	1.3	0.6	1.7	0.8	2.6	0.6	**1.3**
	Chloride	mg/l	20	14	15	17	16	15	**16**
	Phosphorus	µg/l	9.1	9.2	6.6	11.4	7.4	10.2	**9.0**
	Nitrate	mg/l	1.7	1.9	<1	<1	<1	-	**0.7**
	Nitrite	mg/l	<0.005	0.011	<0.005	0.007	<0.005	<0.005	**0.003**
	Sulfate	mg/l	22	18	13	14	11	18	**16**
A2	Temp.	°C	10.5	11.8	13.9	17.7	16.2	15.3	**14.2**
	pH	-	-	-	-	-	-	-	**7.2**
	EC	µS/cm	511	489	509	505	485	492	**499**
	DOC	mg/l C	-	-	-	1.3	1.3	1.1	**1.2**
	Oxygen	mg/l	1.21	2.06	2.58	4.20	3.35	1.84	**2.54**
	Iron	µg/l	0.2	1.4	0.7	0.4	0.4	0.9	**0.7**
	Chloride	mg/l	18	15	17	17	16	17	**17**
	Phosphorus	µg/l	7.1	6.5	5.5	9.8	6.0	6.9	**7.0**
	Nitrate	mg/l	1.2	2.0	<1	<1	<1	<1	**0.5**
	Nitrite	mg/l	<0.005	0.010	<0.005	<0.005	<0.005	<0.005	**0.002**
	Sulfate	mg/l	24	22	23	21	21	22	**22**
Well A	Temp.	°C	11.2	12.5	14.0	15.2	15.6	15.1	**13.9**
	pH	-	-	-	-	-	-	-	**7.7**
	EC	µS/cm	477	464	472	475	463	469	**470**
	DOC	mg/l C	-	-	-	1.3	1.2	1.1	**1.2**
	Oxygen	mg/l	3.87	3.60	3.49	3.08	3.32	3.56	**3.49**
	Iron	µg/l	3.9	2.7	3.2	5.5	9.9	9.4	**5.8**
	Chloride	mg/l	20	16	17	17	17	16	**17**
	Phosphorus	µg/l	10.2	10.0	9.2	11.9	10.8	11.0	**10.5**
	Nitrate	mg/l	2.7	2.6	1.800	1.000	1.000	1.6	**1.8**
	Nitrite	mg/l	<0.005	0.010	<0.005	<0.005	<0.005	<0.005	**0.002**
	Sulfate	mg/l	24	19	20	20	20	21	**21**

Site B[b]

	Parameter	Unit	04/06/19	03/07/19	31/07/19	27/08/19	24/09/19	29/10/19	Average
Kamp river	Temp.	°C	-	-	-	-	-	-	-
	EC	µS/cm	219	352	283	304	417	395	**328**
	DOC	mg/l C	-	-	-	5.1	4.7	4.6	**4.8**
	Oxygen	mg/l	-	-	-	-	-	-	-
	Iron	µg/l	338	201	170	595	190	63	**259**
	Chloride	mg/l	18	33	21	16	27	35	**25**
	Phosphorus	µg/l	74	132	71	52	28	52	**68**
	Nitrate	mg/l	16	12	12	11	10	11	**12**
	Nitrite	mg/l	0.060	0.110	0.024	0.099	0.018	0.470	**0.130**
	Sulfate	mg/l	20	28	20	17	24	28	**23**
B1	Temp.	°C	13.8	13.1	13.7	11.5	12.4	12	**12.8**
	EC	µS/cm	367	372	365	375	320	369	**361**
	DOC	mg/l C	-	-	-	2.3	2.2	2.1	**2.2**
	Oxygen	mg/l	1.11	1.93	1.94	0.12	0.17	1.37	**1.11**

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	Iron	µg/l	0.5	1.0	37.1	0.3	1.0	0.3	**6.7**
	Chloride	mg/l	26	25	24	24	25	25	**25**
	Phosphorus	µg/l	70	65	72	71	67	69	**69**
	Nitrate	mg/l	15.0	16.0	15.0	12.0	10.0	6.7	**12.5**
	Nitrite	mg/l	0.006	0.017	0.630	<0.005	0.440	0.460	**0.259**
	Sulfate	mg/l	23	22	22	21	21	22	**22**
B2	**Temp.**	°C	15.3	12.8	13.3	12.3	12.6	12.2	**13.1**
	EC	µS/cm	440	440	448	461	366	450	**434**
	DOC	mg/l C	-	-	-	1.8	1.5	1.5	**1.6**
	Oxygen	mg/l	7.89	8.70	8.88	10.39	11.40	11.29	**9.76**
	Iron	µg/l	3759	423	292	194	207	298	**862**
	Chloride	mg/l	28	28	29	29	28	27	**28**
	Phosphorus	µg/l	998	81	51	87	44	33	**215**
	Nitrate	mg/l	9.9	13.0	12.0	10.0	11.0	11.0	**11.2**
	Nitrite	mg/l	0.012	0.019	<0.005	0.014	<0.005	<0.005	**0.008**
	Sulfate	mg/l	24	25	24	25	25	24	**24**
Well B	**Temp.**	°C	11.8	12.3	12.5	12.6	12.5	12.6	**12.4**
	pH	-	7.6	6.0	6.1	6.4	6.3	6.2	**6.4**
	EC	µS/cm	437	449	452	463	478	434	**452**
	DOC	mg/l C	-	-	-	1.6	1.6	1.5	**1.6**
	Oxygen	mg/l	6.25	3.80	6.80	6.67	5.93	6.95	**6.07**
	Iron	µg/l	0.4	0.3	0.1	0.2	0.3	0.1	**0.2**
	Chloride	mg/l	28	27	29	29	28	28	**28**
	Phosphorus	µg/l	47.6	48.5	54.8	57.3	54.2	57.2	**53.3**
	Nitrate	mg/l	11.0	13.0	12.0	11.0	11.0	10.0	**11.3**
	Nitrite	mg/l	0.006	<0.005	<0.005	<0.005	<0.005	<0.005	**0.001**
	Sulfate	mg/l	24	22	24	24	24	24	**24**

[a] pH values were not measured in this study period, so an average of the two years before is given.
[b] no pH values available for Site B.

At both sites, the conditions in the subsurface at the first piezometer were suboxic (below 2.5 mg/l oxygen) (Hornstra et al. 2018), leading to significant reduction of iron concentrations after infiltration into the riverbank. However, in piezometer B2 at Site B, iron concentrations increased over 2 orders of magnitude (2 logs) after oxygenation, which was only reflected moderately in the IRB results, and only at the dates 02.07, 30.07 and 29.10 (Fig. 4.3). This may have been an effect of suspended solids in the samples, as mentioned earlier. Possibly these suspended solids were present due to the oxygenation happening close to the sampling location, or suboptimal sampling due to problems with the piezometer. Towards Well B, iron concentrations decreased again, possibly due to the mixing of water from different sources (Fig. 4.1), which may explain the decrease of IRB bacteria at that location.

Sulfate concentrations did not change after oxygenation at Site B, even though concentrations of SRB bacteria increased between B1 and B2 (Fig. 4.3). Unlike iron, sulfate was very stable at ~20 mg/l across all sampling sites, including surface waters. This might explain the overall lower ACs measured by SRB-BART as opposed to IRB-BART, as seemingly

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there were no biogeochemical processes happening that consumed sulfate, at least not in measurable amounts, while this did seem to be the case for iron.

DOC was determined, among other reasons, because the SLYM bacteria's excreted polymer substance (i.e., "slime") consists of a multitude of organic matters. DOC decreased slightly towards the pumping wells at both sites, which might explain the lower concentrations of SLYM bacteria at Well A and B compared to the piezometers. However, the bioavailable part of DOC would be relevant in this case, but this was not determined. Oxygenation between B1 and B2 did seem to increase activity of SLYM bacteria, even though it was only observable during the first 3 sampling dates. The formation of slimes is a complex, multi-parameter occurrence, and is very difficult to be predicted from the presence or absence of one or two parameters. Still, as discussed above, Site B had a less stable chemistry than Site A, which might explain the difference in SLYM bacteria concentrations between the two sites.

The low concentrations of nitrate (NO_3^-) in the backwater and Kamp River might explain why no significant ACs of denitrifying bacteria (DN) were detected by LAB-BART in any of the locations at either site (Fig. 4.3), the mean concentrations were 5 mg/l and 12 mg/l at Site A and B, respectively, while the acceptable limit is 50 mg/l for drinking water (EU, 2020). At Site A, nitrate levels were lower in A1 than in the surface water, indicating denitrifying processes happening during bank filtration, but no subsequent changes in nitrate concentration were observed between A1, A2 and Well A, which explains low concentrations of DN bacteria at these locations. Oxygenation should inhibit the anaerobic respiration of these bacteria, but because they were not present before oxygenation, no effect was observed. The few samples where the AC of DN bacteria was higher (i.e., 15/10/19 at A2, 24/09/19 at Well B, or 29/10/19 at B1), did not show increased concentrations of nitrate or nitrite, so it is difficult to explain why in these samples DN bacteria were more active.

4.3.3 Results of microbial community analysis and detection of LAB-BART relevant physiological bacterial groups

Genome analysis of the samples using metabarcoding revealed the presence of Amplicon Sequence Variants (ASV) associated with approximately 900 bacteria taxa. An average of 38.8% of the taxa were classified as unknown while the remainder indicated the presence of environmental bacteria commonly found in comparable groundwater environments (Yan et

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al., 2021). On a phylum level, Proteobacteria and the recently defined and common groundwater superphylum Patescibacteria have been observed predominantly in all samples (Fig. 4.4) (Tian et al., 2020).

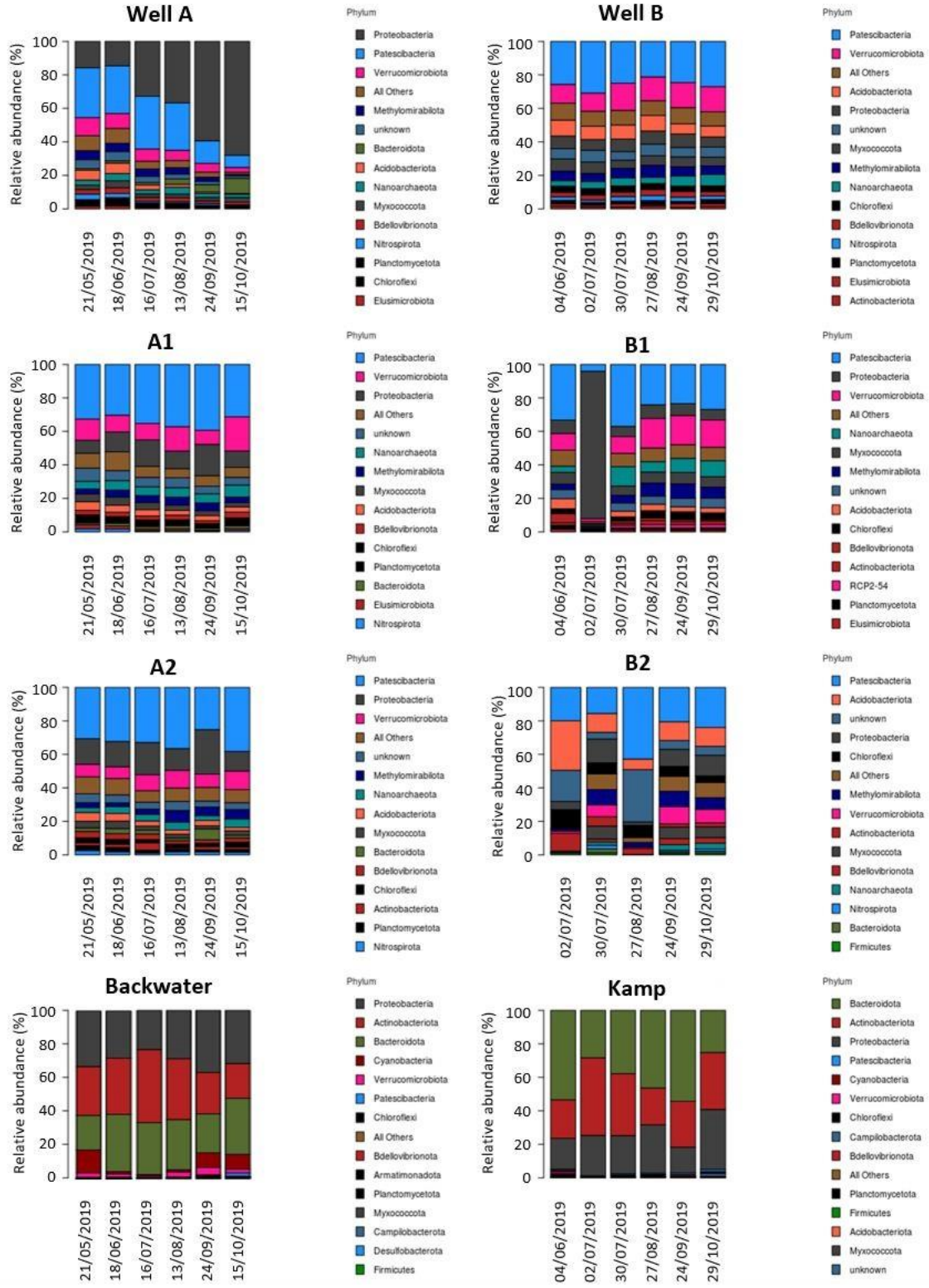

Figure 4.4. Relative abundance at the phylum level in all the samples.

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In recent literature, SRB, IRB, and DN bacteria have been identified as particularly relevant in well fields like the ones under investigation here and are part of the Proteobacteria phylum which is abundant in all samples. As described by Wargin et al. (2007), SRB representatives like *Desulfovibrio* and *Desulfotomaculum* tend to form bacterial consortia negatively influencing organoleptic water quality in groundwater and drinking water distribution infrastructure. Ferric-iron reducers *Geobacter* and *Geothrix* have recently been identified in the USA as notable in the presence of SRB (Flynn et al., 2013) while bacteria capable of denitrification are widely present in groundwater biomes with *Woesearchaeota*, *Nitrospirales*, *Nitrosopumilales* and *Acidobacter* among the most abundant in groundwater used for agricultural purposes (Korbel et al., 2022). All indicators for SRB, IRB and DN bacteria have been observed in at least one replicate sample, and common IRB groundwater genera *Rhodoferax* and *Geobacter* have also been identified as present in the study sites (Flynn et al., 2013; Zaa et al., 2010). While gene metabarcoding does not allow for a quantification of the ASV identified, positive hits in samples that are also positive in the respective LAB-BART strongly indicates true positive results.

4.5 Conclusions

Based on the findings presented here, we found that the LAB-BART test kits are sufficiently consistent and specific for on-site monitoring of drinking water wells. In combination with the fact that gene metabarcoding confirmed that results obtained by LAB-BART did not result in any “false negative” reads, the potential operational hinderances associated with its usage should be minimal. Because LAB-BART only detects active bacteria, it might be useful in specific situations, for example when the presence of viable but not culturable (VBNC) bacteria is known. The kits were found to be simple to handle by professionals without a microbiological background or access to a laboratory. This ease of use is promising for water management professionals in low resources settings worldwide.

At Site B, LAB-BART results were less stable and showed higher fluctuations between sampling locations than at Site A, something that was only moderately shown by the chemical results and not by gene metabarcoding. Possibly, LAB-BART functions better in hydrologically less complicated systems. Mixing of groundwater from different sources and

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the biochemical instability caused by oxygenation can lead to considerable changes in the microbiological communities in the groundwater, which could be difficult to accurately determine. A loss in water production at a simple RBF site, like Site A, would be an exemplary situation where LAB-BART could be beneficial, at least for a preliminary investigation into the type of problem that is causing well clogging. Because of this, in-depth hydrological knowledge of the study site is advised, as it would be difficult to interpret the results otherwise. However, a longer time series of samples would be beneficial, especially when changes take place at the study site, e.g., changes in chemical conditions or pumping rate. This would help to understand how the bacterial communities behave under different stressors such as oxygenation or flow rate changes.

LAB-BART has its limitations. It is very difficult to measure a specific subset of bacteria accurately. Especially if particular taxa are of interest, other quick tests such as the Analytical Profile Index (API) or assays indicating the metabolic activity of fecal bacteria (i.e., *Escherichia coli* or Enterococcus via IDEXX) tests should be employed (Logan et al., 1985; Paziak-Domańska et al., 1999). For more detailed chemical information, such as determining the biochemical conditions in the subsurface, measurements such as oxygen content and photometrics are paramount. Still, as a tool to help characterize well biofouling, LAB-BART could be beneficial if not a substitute for a full microbiological analysis. Although it can give some information on the type of bacteria present in the groundwater, its strength lies in the combination of chemical and microbial processes.

Although LAB-BART results were reasonable when compared to chemical and sequencing data, no definitive verification could be made of the approximate concentration of bacteria that LAB-BART claims to be able to measure. More research is therefore needed, preferably utilizing long term sampling campaigns under more tightly controlled conditions, and with an extended array of molecular and culture-based methods so that the approximate concentration of bacteria can be verified more accurately.

This chapter was based on the following publication:

Oudega, T.J., Leifels, M., Steinbacher, S., Kandler, W., Derx, J., Farnleitner, A., Kirschner, A., Paul, A., 2023. Evaluating a robust and easy-to-use biological-activity- based method to assess the presence of potentially adverse bacteria at two riverbank filtration sites

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along the Danube river : A case study. Österr Wasser- und Abfallw. 1-10 https://doi.org/10.1007/s00506-023-00987-5

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5 Overall Conclusions and Suggestions for Further Work

The goal of this doctoral thesis was to gain insight into the processes and parameters influencing microbial subsurface transport and how this knowledge can be used to define adequate well protection zones. This goal was divided into three main questions:

- To assess an aquifer's capability to reduce pathogens during subsurface flow, tracer tests are often done in columns instead of in the field. Column tests are easier and quicker but often lead to inaccurate results because of the difference in scale, which is why upscaling methods need to be developed. What are the most important parameters and processes that influence upscaling of microbial transport in porous media from the column to the field scale?
- In many countries worldwide, setback distances between a drinking water well and river in an RBF system are calculated by means of the 60- or 50-day travel distance. However, the WHO has stipulated that we should move to a QMRA approach instead, and ensure that the risk of infection stays below 10^{-4} infections/person/year for a given drinking water production site (World Health Organization, 2017a). How does the size of well protection zones change when moving from one method to another, with regards to the pathogens *Cryptosporidium* and *Campylobacter*? What influence does pumping rate play on the setback distance?
- Correct assessment of microbiological groundwater quality is vital for understanding RBF systems and ensuring safe drinking water. A microbiological assay called LAB-BART was tested for its accuracy and ease of use. What are the best use cases for such tests, and how can they be employed in conjunction with conventional microbiological methods to help us to better understand our groundwater systems?

These questions were addressed in three chapters. Chapter 2 investigated upscaling of microbial transport by performing tracer tests on the column and the field scale with two microbial surrogates, namely *B. subtilis* and phiX174. It was found that for both surrogates,

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the parameters that quantify removal rate, such as λ, K_{att}, or α, were 1–2 orders of magnitude higher during the column tests than during the field tests. When compared to other studies that compared column tests to field tests, it was found that the disparity between λ_{column} and λ_{field} was affected most by the type of aquifer, and especially by the amount of preferential flow in the aquifer being studied. For example, a cracked karst aquifer had a disparity of 3 orders of magnitude, while in a homogeneous dune sand aquifer, this value was less than 1 order of magnitude. Important to note here is that between these studies, there were differences in transport distance. The aforementioned karst aquifer tests were done over 97 m, while the dune sand aquifer tests were done over 3.8 m. However, the flow path lengths of these field studies were probably chosen so that it could capture flow elements typical of the aquifer, i.e., crack networks or lenses of course material. Therefore, even though some bias might exist due to transport distance, heterogeneity of the material may still be one of the more important parameters affecting the $\lambda_{column}/\lambda_{field}$ ratio.

In Chapter 3, two different methods for calculating a safe setback distance between a drinking water well and a site of potential contamination were compared, namely the 60-day travel time versus a QMRA approach. In addition, it was studied how changing the pumping rate of the well would influence the setback distances. Additional tracer tests were done at 1 l/s and 10 l/s, using *B. subtilis* as a surrogate for *Cryptosporidium* and *Campylobacter*. It was found that increasing the pumping rate by ten-fold would impact the setback distance with both methods, but not as significantly as expected, as the increase in setback distance was less than 2 times, while in the Austrian regulation this increase was up to 3.8 times. Furthermore, in the case of this specific study site, the setback distance did not change much from one method to the other. However, it was found that when certain input parameters were obtained from the literature (e.g. λ), for example because it would have been too difficult or expensive to measure them at the site, this would lead to a drastic increase in the setback distance for the QMRA method, as it requires a great amount of input data, because conservative values should be chosen based on the soil characteristics. Finally, it was reestablished that scale was a crucial consideration for QMRA as removal rates are scale-dependent and have a large influence on the final results.

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In Chapter 4, the semi-quantitative microbiological assay LAB-BART was used to measure samples taken monthly from two RBF sites along the river Danube in Austria for a total period of six months. The focus was on four different bacterial communities, namely iron-related, sulfate-reducing, denitrifying, and slime-producing bacteria. The results were compared to the chemical composition of the groundwater in addition to gene metabarcoding to validate the suitability of LAB-BART by identifying relevant bacteria, which showed that there were no "false negative" reads. It was found that the LAB-BART system is easy to handle by professionals without a microbiological background or access to a laboratory, and is therefore promising for water management in low resource settings. Its limitations relate to measuring specific subsets of bacteria accurately, and therefore it is not apparent what risks are associated with positive reads, in addition to uncertainty about the approximate concentration of bacteria that LAB-BART claims to measure. For these reasons, it is best used as a tool to characterize biofouling of groundwater well filters.

These conclusions help highlight the challenges that need to be overcome to facilitate a better understanding microbial transport in groundwater to reduce the risk of waterborne disease outbreaks, and provide insight into possible future solutions, such as the upscaling of microbial subsurface transport. The implication of this work is that the discrepancy between the removal rates on the column scale versus the field scale is most affected by the heterogeneity and amount of preferential flow in the porous material studied. This thesis also adds to the understanding of how changing to a QMRA-based approach for establishing safe groundwater protection zones for drinking water wells, will affect the size of these zones, as well as showing the importance of scale aspects in establishing safe well protection zones. By doing so, the results of this thesis lead to a better understanding of the different processes in hydrological systems, which is necessary to ensure safe drinking water. By presenting the differences between aquifers of different kinds, at different places and at different scales, this work demonstrates that it is difficult to establish a generic model or one-size-fits-all method to monitor groundwater and design systems that reduce risks of waterborne disease outbreaks to acceptable levels. Thus, this study once again underlines the understanding that experimental work, especially on the field scale, is vital for achieving

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safe drinking water. This is especially true in the case of performing QMRA to calculate reliable setback distances, as parameters that govern pathogen reduction during subsurface flow, such as λ or K_{att}, but also the rate of inactivation, μ, have a significant effect on the accuracy of such calculations.

Future research should focus on experimental work. By continuously adding data from more and more aquifers, soil types, and hydrological systems to the literature, and combining this with numerical models, we will be able to better understand and eventually develop reliable upscaling methods for microbial transport. It is important to clearly define objectives as a community and couple this with extensive literature review to identify gaps in knowledge. The focus of future tests should then be on those gaps. It is important to test a variety of microbial surrogates under different conditions, whether chemical (e.g. oxic versus anoxic waters), lithological (e.g. carbonate versus silicate porous media), or structural (e.g. cracked versus uncracked aquifers). This last point is vital as this thesis has shown that aquifer structure is a major component affecting pathogen transport. We need to do more research both in the field and in columns, but this research also needs to include the mesoscale, for example, in large, undisturbed columns. Besides that, the focus should lie on developing models that can work at several scales. To do this, we need to know why scale differences exist, which parameters change due to scale and why, so these processes can be included in such models. Only then can we eventually reach a situation in which we can confidently predict pathogen concentrations in many groundwater systems worldwide. In conclusion, there is no substitute for experimental work, especially on the field scale, and coupling this with small scale and mesoscale experiments can lead to a better understanding of microbial transport processes, which in turn should lead to the development of drinking water management systems with lower risk of waterborne disease outbreaks.

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