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DOI
10.1016/j.watres.2022.118724

Publication date
2022

Document Version
Final published version

Published in
Water Research

Citation (APA)
Eisfeld, C., Schijven, J. F., van der Wolf, J. M., Medema, G., Kruisdijk, E., & van Breukelen, B. M. (2022). Removal of bacterial plant pathogens in columns filled with quartz and natural sediments under anoxic and oxygenated conditions. *Water Research*, *220*, 1-10. Article 118724. https://doi.org/10.1016/j.watres.2022.118724

Water Research 220 (2022) 118724

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Removal of bacterial plant pathogens in columns filled with quartz and natural sediments under anoxic and oxygenated conditions

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ARTICLE INFO

Keywords:
Plant pathogen transport
Water quality
Groundwater contamination
Managed aquifer recharge
Irrigation
Aquifer recharge and recovery

ABSTRACT

Irrigation with surface water carrying plant pathogens poses a risk for agriculture. Managed aquifer recharge enhances fresh water availability while simultaneously it may reduce the risk of plant diseases by removal of pathogens during aquifer passage. We compared the transport of three plant pathogenic bacteria with *Escherichia coli* WR1 as reference strain in saturated laboratory column experiments filled with quartz sand, or sandy aquifer sediments. *E. coli* showed the highest removal, followed by *Pectobacterium carotovorum, Dickeya solani* and *Ralstonia solanacearum.* Bacterial and non-reactive tracer breakthrough curves were fitted with Hydrus-1D and compared with colloid filtration theory (CFT). Bacterial attachment to fine and medium aquifer sand under anoxic conditions was highest with attachment rates of max. k_{att1} = 765 day^{-1} and 355 day^{-1}, respectively. Attachment was the least to quartz sand under oxic conditions (k_{att1} = 61 day^{-1}). In CFT, sticking efficiencies were higher in aquifer than in quartz sand but there was no differentiation between fine and medium aquifer sand. Overall removal ranged between < 6.8 log$_{10}$ m^{-1} in quartz and up to 40 log$_{10}$ m^{-1} in fine aquifer sand. Oxygenation of the anoxic aquifer sediments for two weeks with oxic influent water decreased the removal. The results highlight the potential of natural sand filtration to sufficiently remove plant pathogenic bacteria during aquifer storage.

Abbreviations

AIC	Akaike Information Criterion
BTC	breakthrough curve
CFT	colloid filtration theory
EC	electrical conductivity
MAR	managed aquifer recharge
SSF	slow sand filtration

1. Introduction

Fresh water availability is decreasing as consequence of overuse and extreme weather events. With agriculture being the biggest water consumer claiming about 70% of the total fresh water, it relies heavily on the advancement of water management practices (UN-Water, 2021). One strategy to tackle fresh water availability in agricultural settings is managed aquifer recharge (MAR) which includes riverbank filtration, infiltration ponds, or aquifer storage and recovery (Dillon, 2005). The goal is to replenish aquifers and improve water quality as microbial pathogens are removed during soil filtration. Removal is crucial because surface waters used for MAR may contain pathogenic organisms. Their number should decline to safe levels prior to irrigation to avoid the spread of (plant) diseases (Hong and Moorman, 2005). Chemical, biological, and physical processes occurring at the soil-water interphase during MAR can remove pathogens (Ginn et al., 2002). This includes physical straining, die-off in the water phase, (ir)reversible attachment to sediment grains, die-off at the grain surface, and die-off through predation and competition with other microorganisms (Bradford et al.,

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https://doi.org/10.1016/j.watres.2022.118724
Received 24 February 2022; Received in revised form 1 June 2022; Accepted 6 June 2022
Available online 7 June 2022

2013).

Several studies analyzed the removal efficiency of MAR systems focusing on human pathogens and related human health risks after irrigation of crops or recreational areas (e.g. Ayuso-Gabella et al., 2011; Page et al., 2010; Toze, 2004). In contrast, no study investigated the removal of plant pathogens during MAR, although MAR treated water is often intended for irrigation and related consequences for plant health have high ecological and economic value (Hong and Moorman, 2005; Savary et al., 2019). For example, surface water near potato or tomato growing fields must be tested for the presence of *R. solanacearum* and an irrigation ban is imposed in areas with contaminated surface water (Anonymous, 1998). Water treatment of potentially contaminated surface water via MAR is currently not considered in legislations to provide safe irrigation water. Legislations that regulate water reuse account for microbiological water quality but focus currently only on human health risks and neglect plant pathogens and related plant health (Anonymous, 2020; Council, 2009). Risk management is an essential component of a successful water reuse scheme but requires quantitative information on pathogen removal to analyze microbiological water quality. This information is currently lacking for plant pathogens.

To our knowledge, only two studies investigated the fate of plant pathogens in saturated porous media. Liu et al. (2008) showed that biofilm coated glass beads increased the removal of the bacterium *Erwinia chrysanthemi*. Jeon et al. (2016) demonstrated a higher retention of zoospores of the oomycete *Phytophthora capsici* in iron-oxide coated saturated quartz sand in comparison to uncoated sand. Both studies used "fabricated", homogenous sediment to study principal transport processes. Natural sediments are heterogeneous in their lithological and chemical composition. They usually contain positively charged metal-oxide coatings that increase the number of favorable attachment sites and enhance the removal of the negatively charged microorganisms (Bradford et al., 2013; Johnson et al., 1996). Moreover, MAR usually results in oxygenation of aquifers and may lead to formation of additional iron-oxides. Studies on transport of microbial plant pathogens in natural sediments are only available within the context of slow sand filtration (SSF) (e.g. Ferreira et al., 2012; Lee and Oki, 2013) which show a high efficiency to remove pathogens from greenhouse irrigation waters. For example, a horizontal slow sand filter with a length of 27.6 m removed 99.5% of *Fusarium* propagules (Prenafeta-Boldú et al., 2017). However, SSF and MAR differ in hydrological and geochemical conditions in terms of lithology, water saturation, redox conditions, and biological activity which makes a comparison of both systems difficult.

The aim of our study was to analyze the removal of the following three economically and ecologically important plant pathogenic bacteria during transport in laboratory columns filled with aquifer sediments (or quartz) for the first time: *Ralstonia solanacearum* (brown rot), and the soft rot Pectobacteriaceae (SRP) *Dickeya solani* and *Pectobacterium carotovorum*. These bacteria all cause high crop losses to a broad variety of crops including potato and tomato but also can affect ornamental flower production (Hayward, 1991; Ma et al., 2007; Tjou-Tam-Sin et al., 2016; van der Wolf and De Boer, 2007). We compare their transport with *Escherichia coli* WR1 which has been used in previous field (Schijven et al., 2000) and column studies with natural sand (Hijnen et al., 2005; Hijnen et al., 2004), but not in quartz sand. The aquifer sediments were obtained from a MAR site used to produce and store irrigation water. We hypothesize that a higher removal is achieved in natural sediment as these contain more favorable attachment sites in comparison to clean quartz sand. Furthermore, the effect of oxygenation of the naturally anoxic sediments on removal rates was tested as this could lead to the formation of additional positively charged iron oxides. The resulting breakthrough curves (BTC) of the column experiments were modelled in Hydrus-1D using the advection-dispersion equation extended with a 1- or 2-site kinetic attachment/detachment model (Schijven et al., 2002; Schijven and Šimůnek, 2002). Log removals per meter were calculated to estimate removal under field conditions and for comparison with other studies (Pang, 2009).

2. Material and methods

2.1. Porous media and aqueous solutions

Fine to medium grained silica quartz sand (d_{50} = 260 μm) with a 99.5% SiO_2 content (M32, Sibelco, Belgium) was used in the quartz sand experiments. To remove any metal oxides the sand was acid-washed (Chu et al., 2001) which we describe in the supplement (S1). For experiments with aquifer sand, sediment was obtained during the drilling operation of a MAR site in Breezand, the Netherlands (coordinates: 52.8883, 4.8221). The semiconfined sandy aquifer (11.5-33.0 m below surface level) of late Holocene and Pleistocene age lies below a confining Holocene clay/peat layer. Sediment samples were obtained from the boreholes using a 2 m sonic drill aqualock system with a core catcher. The sand cores were confined on both ends and stored in closed PVC tubes at 4 °C for one month before they were opened for sand sample collection. After opening, the samples were exposed to air but handled rapidly to avoid oxidation. Two soil samples from different depths were collected with a disinfected (70% EtOH) spoon for the column experiments: (i) fine aquifer sand from 12-14 m depth with d_{50} = 192 μm, and (ii) medium aquifer sand of d_{50} = 305 μm from 24 m depth. The coefficient of uniformity ($C_u = d_{60}/d_{10}$) is 1.6 for quartz sand, 2.0 for fine and 2.3 for medium MAR sand. A $C_u < 2$ refers to uniform soils. A grain size distribution chart is shown in Fig. S1 and Table S1 together with a chemical soil analysis in Table S2. Both soil samples are representative for the aquifer which consists to over 80% of fine to medium coarse sand (125-500 μm). The soil samples were placed in a zip-locked plastic bag of which excess air was removed before closure, and stored in a gas tight container initially flushed with N_2 gas together with two anaerobic gas generation sachets (Oxoid™ AnaeroGen™ 2.5 L, Thermo Fisher Scientific). The container was stored at 10 °C in the dark until further use. All three sand types (quartz, fine and medium aquifer) were extracted for total iron using the citrate-dithionite extraction method after Claff et al. (2010) and analyzed using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

An artificial groundwater (aGW) solution was prepared in autoclaved containers after Bolster et al. (2001) and is described in S1. The aGW was selected to exclude variations in bacteria attachment resulting from variations in solution chemistry. It is not identical to the MAR groundwater but contains the major ions and has low ionic strength of about 3 mM which does not favor attachment (Foppen and Schijven, 2006). For the non-reactive salt tracer solution 1 g L^{-1} sodium chloride was added to the aGW. For all anoxic experiments, the aGW or tracer solution were made anoxic by flushing about 2 L with nitrogen gas for 1 h.

2.2. Column setup

Recommendations by Gilbert et al. (2014) were followed for the dimensioning of the column and selection of materials in the experimental setup (setup shown in Fig. S2). We used a PVC column with a length of 23 cm and an inner diameter of 3.6 cm. Therefore, the representative elementary volume (REV) calculated by dividing the column diameter by the grain diameter results in a REV of 128 for quartz, 168 for fine MAR and 105 for medium MAR sand. These values are all higher than the minimum REV of 40 for microbiological experiments. The column was wet-packed by pouring the sand material into the column and applying a slow flow rate (<0.5 mL min^{-1}) of anoxic aGW with bottom-up flux to avoid air entrapment. Additionally, the column was continuously tapped while filling to remove air bubbles which required specific attention with natural sand. A metal mesh (100 μm pore size) was placed at the top and bottom of the column to retain the sand and was reinforced by a perforated metal plate (1 mm thick, 2 mm wide holes). Column packing was executed under aerobic conditions while flushing with N_2 at the column headspace and pumping anoxic aGW from bottom-up. The tubing (polyurethane) was used with appropriate

connectors (Festo B.V., The Netherlands) and all materials were autoclaved prior to use. The column was rinsed carefully with 70% EtOH prior to use. A peristaltic pump (Watson Marlow 205U, UK) maintained a constant pump rate of 2.5 mL min^{-1} (± 0.1 mL min^{-1}) which was confirmed by logging the effluent gravitationally (Kern & Sohn GmbH, Germany). The velocity of 3.6 m day^{-1} was chosen according to an average velocity of 1.4-5.3 m day^{-1} within the MAR system between infiltration and abstraction well, considering only radial horizontal water flow in the most permeable layer of the aquifer. The velocity is higher next to the infiltration point and decreases with distance towards the extraction well located at 6 m distance. An oxygen flow-through cell (PreSens, Germany) at the column in- and outlet monitored the oxygen concentrations automatically. Additionally, a flow-through EC sensor at the outlet measured the EC in the effluent with a datalogger (WTW, Xylem Analytics Germany) to monitor the breakthrough curves of the non-reactive salt tracer. We conducted three experiments with different saturated porous media which are summarized in Table 1: (E1) clean quartz sand under oxic conditions, serving as worst-case scenario and reference experiment, conducted with all bacteria; (E2) fine aquifer sand under both anoxic and oxygenated conditions with *P. carotovorum*; (E3a) medium aquifer sand under anoxic conditions done with *R. solanacearum, E. coli*, and *P. carotovorum*, and (E3b) oxygenated medium aquifer sand with *P. carotovorum*. Experiments with quartz sand were conducted in duplicates and with freshly packed columns, while the experiments with fine aquifer sand were only done once per condition, but using the same column to subsequently test oxygenation. The experiments with medium aquifer sand were performed in triplicates using the same column, but were freshly packed for each bacterium. The transport of *P. carotovorum* in medium MAR sand was first studied under anoxic conditions, followed by oxygenation of the same column to study the transport under oxic conditions. Thereby, only one parameter, namely the influence of oxygen, was changed. In between the replicate experiments, the column was flushed (1.5 mL min^{-1}) with anoxic deionized water over night to remove attached bacteria before the column was equilibrated again with aGW. At the beginning of the bacterial breakthrough experiments, an effluent sample was taken shortly after the start of the pathogen loading when bacterial breakthrough was not yet expected. The effluent sample was analyzed for the presence of the selected pathogen by dilution plating. Oxygenation of the column with fine aquifer sand (E2) was executed by flushing (1.5 mL min^{-1}) the column for two days with oxic aGW until the effluent O_2 concentration remained stable. This flushing was extended to two weeks in experiments with medium aquifer sand (E3b) to achieve more extensive oxidation of the sediments and potentially more formation of reactive surface metal oxide coatings. All experiments were performed in a cooling chamber at 10 °C (± 1.5) except for experiments with *R. solanacearum* which had to be performed within a laminar flow cabinet at room temperature due to the bacteria's quarantine status.

Table 1
Experimental conditions for column experiments in different sand types.

		Saturated porous media	Bacterium	Temp °C	Redox
Silica quartz sand, Acid-washed	E1	fine d_{50} = 260 µm d_{60}/d_{10} = 1.6	*D. solani* *P. carotovorum* *E. coli* *R. solanacearum*	10 (±1.5) [a]RT (23 ±2)	Oxic
	E2	fine, d_{50} = 192 µm d_{60}/d_{10} = 2.0	*P. carotovorum*	10 (±1.5)	Anoxic [b]Oxic
Natural sand from MAR site	E3a	medium d_{50} = 305 µm d_{60}/d_{10} = 2.3	*E. coli* WR1 *R. solanacearum*	10 (±1.5) [a]RT (23 2)	Anoxic
	E3b		*P. carotovorum*	10 (±1.5)	Anoxic [c]Oxic

[a] RT = room temperature
[b] Continous column flushing with oxic artificial groundwater (aGW) for 2 days
[c] Continous column flushing with oxic aGW for 2 weeks

The column was operated in upflow direction at a pump rate of 2.5 mL min^{-1} to ensure saturated conditions. Each experiment was setup as following: (1) Filling of the column with sand material and equilibration with about 13 pore volumes (PVs) aGW. (2) Breakthrough experiments with the non-reactive salt tracer solutions. There, the sodium chloride solution was pumped for 85 min (ca. 2.4 PVs) followed by switching back to the aGW solution until the initial EC was measured. (3) Bacterial breakthrough experiments where the bacterial suspension containing ca. 10^6 CFU mL^{-1} was also pumped for 85 min followed by switching back to the aGW solution. Effluent samples of ca. 0.5 mL were collected manually in reaction tubes at adequate time points and evaluated by dilution plating.

2.3. Inoculation solution

R. solanacearum race 3 biovar 2 (phylotype II) strain IPO-1828, D. solani IPO-2266, and P. carotovorum IPO-1990 are plant pathogenic bacteria present in surface waters and were used in this study. Their transport was compared with Escherichia coli WR1 (NCTC 13167). The selected bacteria are all gram-negative, rod-shaped, flagellated and have a similar size of 1.2-2.5 µm in length and 0.4-1 µm in width. Zeta potentials have been obtained from literature and they show that all pathogens are negatively charged: -9.85 mV for P. carotovorum (Gutierrez-Pacheco et al., 2018), -22 mV for E. coli WR1 (Schijven et al., 2008), -21.3 mV for R. solanacearum (Yan et al., 2004) and -25.03 mV for Dickeya spp., formerly named Erwinia chrysanthemi (Liu et al., 2008). Bacterial suspensions were prepared by incubating the respective bacteria in oxic liquid medium overnight for 15 h at 28°C (E. coli 12 h at 37 °C) on a rotary shaker at 100 rpm. Therefore, the bacteria were harvested during the late log and early stationary growth phase. Details on bacteria and culture media preparation can be found in the supplement (S2). The grown cultures were harvested by centrifugation (3500 x g, 20 min at room temperature), followed by washing and resuspending the pellet in a quarter strength Ringer's solution (Sigma-Aldrich; St. Louis, USA). This pelleting and washing step was repeated twice to remove any excess broth. The bacterial suspension was then diluted to reach an optical density of 0.1 at 600 nm representing a concentration of 108 CFU mL^{-1}, and was used to inoculate 250 mL of (anoxic) aGW to reach a final concentration of 106 CFU mL^{-1}. The concentration of the inoculation suspension (C0) and the inoculated aGW before and at the end of the breakthrough experiment, and enumeration of the bacterial effluent concentrations were confirmed by dilution-plating using selective media (S2) (Elphinstone et al., 1998; Hélias et al., 2012). Furthermore, bacterial hydrophobicity was analyzed using the microbial adhesion to hydrocarbon (MATH) test as described by Gargiulo et al. (2008).

2.4. Data analysis and modeling

Breakthrough curves of the non-reactive salt tracer and bacteria were analyzed with Hydrus-1D (Simunek et al., 2005). Solute transport of the non-reactive tracer is described by the advection-dispersion equation under steady state flow conditions and a constant head:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - \nu\frac{\partial C}{\partial x} \quad (1)$$

where C is the solute concentration in the liquid phase [M L^{-3}], t is time [T], D is the hydrodynamic dispersion coefficient [L^2 T], x is distance [L] and ν the average interstitial water velocity [L T^{-1}]. Bacterial transport is modeled by an extended form of the advection-dispersion equation accounting for two kinetic sites with reversible bacterial attachment and detachment rate coefficients (Schijven et al., 2002):

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - \nu\frac{\partial C}{\partial x} - (k_{att1} + k_{att2})C + k_{det1}S_1\frac{\rho_B}{\theta} + k_{det2}S_2\frac{\rho_B}{\theta} \quad (2)$$

$$\frac{\rho_B}{\theta}\frac{\partial S_1}{\partial t} = k_{att1}C - k_{det1}S_1\frac{\rho_B}{\theta} \quad (3)$$

$$\frac{\rho_B}{\theta}\frac{\partial S_2}{\partial t} = k_{att2}C - k_{det2}S_2\frac{\rho_B}{\theta} \quad (4)$$

where ρ_B is dry bulk density [M L^{-3}]; θ is porosity [-]; S_1 and S_2 are the concentrations of attached bacteria at two kinetic sites [M M^{-1}]. k_{att} and k_{det} [T^{-1}] are the attachment and detachment rate coefficients, respectively, of free and attached microorganisms, while subscripts 1 and 2 refer to the two different kinetic sites. In Hydrus-1D, normalized C/C_0 values were used as input values and to model the tailing of the BTC. Log transformation of C/C_0 was applied to overcome the great difference between maximum and minimum bacterial effluent concentrations (Schijven and Šimůnek, 2002).

Porosity θ and longitudinal dispersivity α_L [L] ($\alpha_L = D/v$) were both determined from salt tracer experiments and used as input values to model bacterial BTC. Growth or die-off of the bacteria was not considered within the time span of the experiments (about 9 h) as we have shown in batch experiments that die-off in the water phase took only place after more than 24 h (Eisfeld et al., 2021). Hydrus-1D allows to compare the fit of a 1- or 2-site kinetic model by evaluating the coefficient of determination R^2 and the Akaike Information Criterion (AIC) as output data from Hydrus-1D. The AIC considers both the goodness of fit and parsimony of the models. For our experiments, the model with the smallest AIC value was therefore chosen as best model.

The advection-dispersion Eq. (2) accounts for reversibility of the bacterial attachment. We compare these results with the CFT that assumes a single grain size with perfect sphericity and smoothness where bacteria attach irreversibly. We used the equations described by Tufenkji and Elimelech (2004) to calculate the single-collector contact efficiency (η_0) and sticking efficiency (α). To derive α, the C/C_0 input values were calculated using the equation described by Schijven et al. (2000):

$$\log\left(\frac{C}{C_0}\right) = \frac{x}{2.3}\frac{\left(1 - \sqrt{1 + 4\alpha_L\frac{k_{att1} + \mu_l}{\nu}}\right)}{2\alpha_L} \quad (5)$$

which employs the Hydrus-1D output values α_L and k_{att1} from the salt and bacterial breakthrough experiments, respectively. Finally, the C/C_0 is used to estimate microbial log-removal per meter expressed by λ [$\log_{10}$ L^{-1}] (Pang, 2009).

3. Results and discussion

3.1. Chloride tracer BTC

The non-reactive salt tracer allowed to determine the physical water flow characteristics of the column. The normalized BTCs of the chloride EC effluent concentrations (C/C_0) were fitted in Hydrus-1D to obtain longitudinal dispersivity α_L and porosity Θ as shown in Table S3. In quartz sand, these parameters were evaluated for each experiment individually and the porosity was 0.37 - 0.40 with α_L of 0.038 - 0.045 cm. The same parameters were obtained in the aquifer sand experiments by combining the data of the chloride BTCs of the replicate experiments per bacterium. For *R. solanacearum* in medium aquifer sand, only two salt tracer tests were executed. The porosity in fine and medium aquifer sand was lower (0.31 - 0.34) than in the quartz sand experiments, whereas α_L was considerably higher ranging between 0.28 – 1.5 cm. α_L of medium aquifer sand used for experiments with *E. coli* was 1.51 cm, thereby similar to the columns filled with fine sand (1.46 cm). Differences in porosity and longitudinal dispersivity may arise from combining data of the tracer BTCs of the aquifer sand, but also from column packing or variation in grain size distribution of the natural aquifer sand (Fig. S1). These variations in dispersivity have also been reported by other authors (Oudega et al., 2021; Schijven et al., 2002) but have little influence on the fitting and adsorption parameters.

3.2. Bacterial transport in quartz sand

The bacterial BTCs are shown in Figs. 1 and 2. The modified advection-dispersion Eq. (2) for bacterial transport implemented in Hydrus-1D was applied to fit the bacterial BTCs. Table 2 shows the attachment and detachment parameters for a 1- or 2-site kinetic model. In most cases, the 2-site kinetic model showed a better fit when comparing the R^2 and AIC (Table S5). All BTCs of quartz and natural MAR sand were analyzed for blocking by fitting the blocking parameter S_{max1} (e.g. Hornstra et al. (2018)) in Hydrus-1D. In blocking, available grain surfaces get occluded by for example attached bacteria which decreases the retention of other bacteria as less favorable sites are available (Ryan and Elimelech, 1996). Adding the blocking parameter did not improve model fit considering the AIC.

In all experiments, the bacterial BTCs coincided with the tracer BTCs, but the bacterial BTCs showed a lower C/C_0 as consequence of bacterial attachment which we describe as removal. Fig. 1 presents the results of the duplicate bacterial BTCs in quartz sand. Additionally, Fig. S3A compares the BTCs of the four bacteria where C/C_0 is presented linearly, while Fig. S3B displays the same results using log-transformed C/C_0 values which allowed to identify the tails of the BTCs caused by detachment. *E. coli* had the highest removal in quartz sand, followed by *P. carotovorum* and *D. solani*. *R. solanacearum* was least removed reflected by a high C/C_0 of 0.85 in replicate 1 and a low k_{att1} of 6 day^{-1}. In comparison, k_{att1} was ten times higher for *E. coli* (61 day^{-1}, replicate 1) and the C/C_0 peak was 0.18. The bacterial BTC of *E. coli* was best fitted with the 1-site kinetic model and therefore, removal is solely guided by kinetic site 1. The BTC of the plant pathogenic bacteria were best fitted with the 2-site kinetic model but a comparison of the AICs often showed little difference indicating that the 1-site model would also be an adequate fit. At kinetic site 2, attachment occurred to a lesser extent than at site 1 (k_{att2} = 1 - 4 day^{-1}). While kinetic site 1 showed little detachment (k_{det1} = 0.03 – 2.2 day^{-1}) detachment at site 2 was very high ranging from 11-112 day^{-1}. Note that k_{att1} of *D. solani* and *R. solanacearum* and all k_{det2} rate coefficients show a very high standard deviation. Its implication will be discussed in the section of parameter evaluation. Overall, the plant pathogenic bacteria were removed to a lesser extent than *E. coli* and their BTC peak had a higher C/C_0 value. Reported bacterial characteristics such as cell size, surface charge or growth stage are similar for the selected bacteria. Moreover, the results of the hydrophobicity test (Table S4) indicate that all bacteria are hydrophilic with little difference (2.1-8.9%) in partitioning into the hydrophobic phase. Therefore, other bacterial characteristics like the specific surface structure (e.g. lipopolysaccharides) may cause the different transport behaviors (Gilbert et al., 1991; Ginn et al., 2002). Nevertheless, these were not further analyzed and should be addressed in future studies.

3.3. Bacterial transport in aquifer sand

Bacterial BTCs in medium aquifer sand are shown in Fig. 2A-D. The replicate experiments for each bacterium show a higher variability in aquifer than in quartz sand as a result of the greater heterogeneity of the sand material represented by a higher C_u. In some experiments, retained bacteria from a previous replicate experiment detached and were measured in the effluent before the actual bacterial breakthrough was expected. Yet, these concentrations were low ($<10^2$ and once 10^3 CFU/mL) and were in the range or lower than the tail concentrations caused by detachment. The peak breakthrough of the bacteria and the overall removal was not influenced. For example, during the transport of *E. coli*

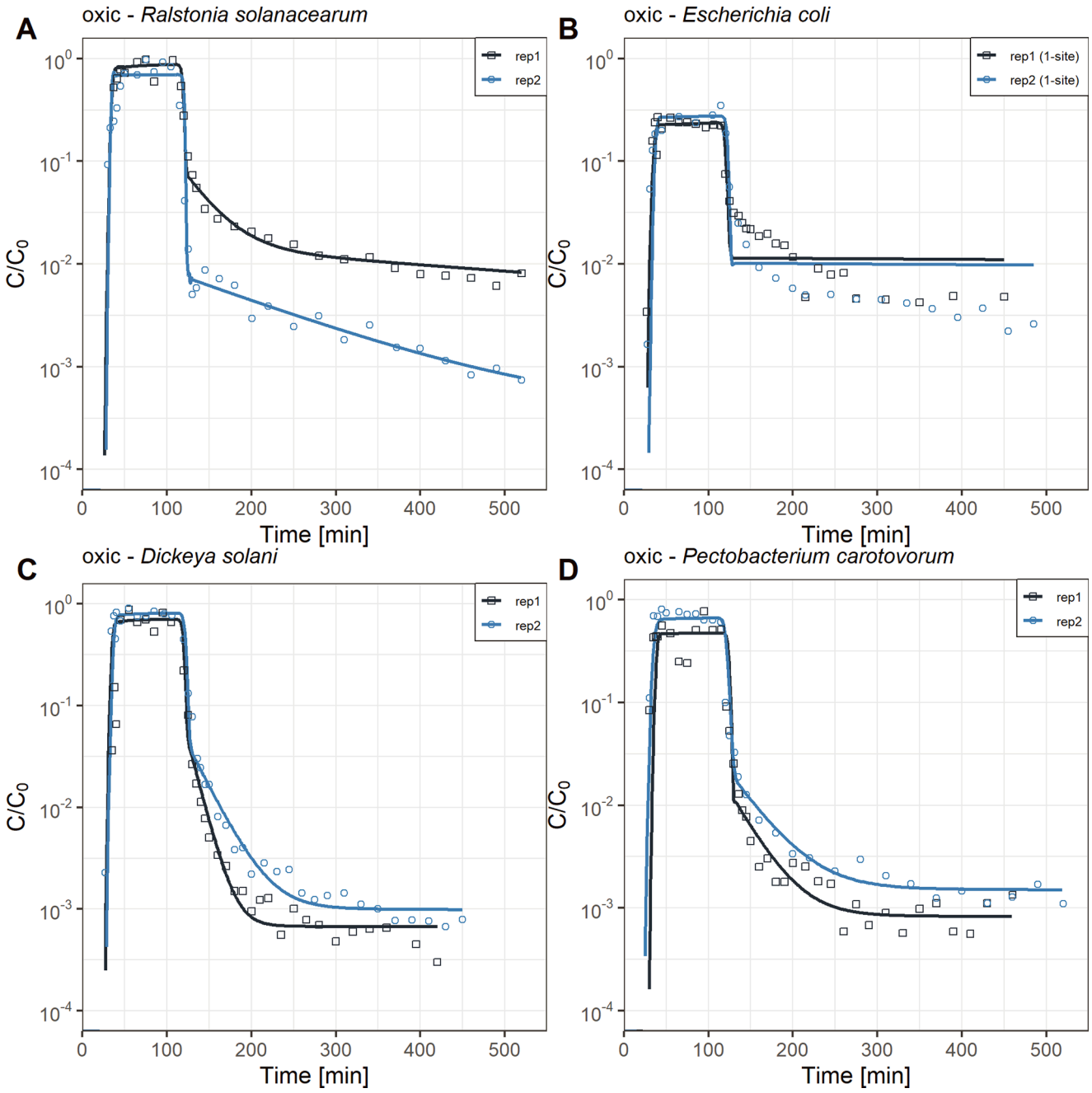

Fig. 1. Duplicate experiments of bacterial breakthrough experiments in quartz sand under oxic conditions (rep = replicate). The normalized effluent concentrations (C/C_0) are plotted as a function of time (min) on a semi-log scale. Solid lines are the fitted models obtained from Hydrus-1D and the symbols are the corresponding experimental data in the same color. All breakthrough curves (BTCs) have been modelled with the 2-site kinetic model, expect for the BTCs of Escherichia coli WR1 which was modelled with the 1-site kinetic model.

in anoxic medium MAR sand (replicate 2 and 3), about 10^2 CFU/mL were detected in the first sample taken after 1 min. However, k_{att1} as well as the overall removal were higher in replicate 2 and 3 in comparison to replicate 1 which did not yet contain attached bacteria.

In anoxic medium aquifer sand, the C/C_0 values were at least three orders of magnitude lower than in the quartz sand. Differences in attachment to quartz and natural MAR sand can also be explained by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Derjaguin and Landau, 1993; Verwey et al., 1948). It describes the repulsive and attractive forces between the bacterial cell and grain surface. Clean quartz sand has a negative surface charge (negative zeta potential) that repulses negatively charged bacteria (Elimelech et al., 2000). Unfavorable attachment can occur when the distance between grain surface and bacterial cells gets small enough and the attractive van der Waals forces dominate over the repulsive double layer. Favorable attachment occurs in natural sediments where metal oxides depict favorable attachment sites that attract negatively charged bacteria. Fine and medium MAR sand had the same amount of Fe- and Al-metal oxides (Table S2) although the total extractable iron was higher in medium aquifer (21 ± 0.6 mg L^{-1}) than in fine aquifer sand (6.8 ± 0.7 mg L^{-1}, Table S6). Grain size played an important role in the retention of the bacteria as the MAR sand has a larger fraction of fine sands compared to quartz providing a greater total surface area for attachment. Likewise, the retention in fine MAR sand has been greater than in medium MAR sand. They varied in the fraction of fine sands smaller than 250 μm which was about 77% in fine and 30% in medium MAR sand. These fractions of very fine sands,

Table 2
Fitting parameters of the bacterial BTC in different sand media. Standard error of the parameters are presented in parentheses.

Bacterial breakthrough experiments		Hydrus-1D results								Calculated results					
		k_{att1}		k_{det1}		k_{att2}		k_{det2}		R^2	C/C_0 peak	η_0	α	λ	
Quartz sand		[day^{-1}]		[day^{-1}]		[day^{-1}]		[day^{-1}]			[-]	[-]	[-]	[log$_{10}$ m^{-1}]	
Pc	rep 1	**28**	(3)	**0.04**	(0.02)	**1**	(1)	**49**	(39)	0.92	0.45	0.009	0.117	2.7	(0.5)
	rep 2	**17**	(16)	**0.09**	(0.08)	**1.4**	(2)	**39**	(36)	0.77	0.63	0.009	0.067		
Ds	rep 1	**15**	(59)	**0.05**	(0.01)	**4**	(2)	**112**	(28)	0.95	0.65	0.011	0.047	1.4	(0.3)
	rep 2	**8**	(9)	**0.10**	(0.02)	**2**	(1)	**57**	(14)	0.95	0.79	0.011	0.027		
Rs	rep 1	**6**	(21)	**2.2**	(1.39)	**4**	(3)	**45**	(47)	0.99	0.85	0.011	0.018	0.42	(0.2)
	rep 2	**1**	(1)	**0.03**	(0.15)	**1**	(1)	**11**	(18)	0.73	0.97	0.011	0.003		
Ec	rep 1 (1-site)	**61**	(2)	**0.58**	(0.30)					0.92	0.18	0.012	0.176	6.8	(0.4)
	rep 2 (1-site)	**51**	(2)	**0.49**	(0.44)					0.9	0.24	0.012	0.149		
Medium aquifer sand (anoxic)															
Pc	rep 1	**184**	(10)	**0.38**	(0.13)	**25**	(6)	**30**	(9)	0.86	$1.2x10^{-2}$	0.009	0.617	19	(1.3)
	rep 2 (1-site)	**129**	(3)	**0.55**	(0.25)					0.88	$3.9x10^{-2}$	0.009	0.451		
	rep 3 (1-site)	**202**	(3)	**0.55**	(0.16)					0.85	$7.9x10^{-3}$	0.009	0.670		
Rs	rep 1	**161**	(8)	**0.03**	(0.04)	**24**	(25)	**33**	(27)	0.9	$1.7x10^{-2}$	0.012	0.424	18	(1.7)
	rep 2	**208**	(4)	**0.25**	(0.19)	**31**	(26)	**25**	(35)	0.94	$6.0x10^{-3}$	0.012	0.534		
	rep 3	**155**	(16)	**0.07**	(0.11)	**25**	(18)	**23**	(22)	0.93	$2.0x10^{-2}$	0.012	0.410		
Ec	rep 1	**355**	(8)	**0.4**	(0.06)	**75**	(8)	**0.4**	(7)	0.91	$4.1x10^{-3}$	0.013	0.540	28	(2)
	rep 2	**551**	(28)	**0.13**	(0.07)	**107**	(25)	**19**	(3)	0.63	$6.3x10^{-4}$	0.013	0.725		
	rep 3	**419**	(8)	**0.26**	(0.08)	**132**	(26)	**79**	(21)	0.84	$2.2x10^{-3}$	0.013	0.604		
Medium aquifer sand (oxygenated)															
Pc	rep 1 (1-site)	**53**	(2)	**0.58**	(0.17)					0.96	$2.41x10^{-1}$	0.01	0.182	13	(4)
	rep 2 (1-site)	**215**	(3)	**1.48**	(0.16)					0.8	$5.78x10^{-3}$	0.01	0.657		
	rep 3 (1-site)	**103**	(5)	**0.75**	(1)					0.69	$7.05x10^{-2}$	0.01	0.338		
Fine aquifer sand															
Pc	anoxic (1-site)	**765**	(91)	**0.91**	(0.79)					0.81	$1.0x10^{-4}$	0.014	0.569	40	
	oxygenated	**515**	(30)	**0.86**	(0.38)	**317**	(415)	**155**	(258)	0.21	$8.1x10^{-4}$	0.014	0.440	31	

Pc = *Pectobacterium carotovorum, Ds* = *Dickeya solani, Rs* = *Ralstonia solanacearum, Ec* = *Escherichia coli* WR1; R^2 = coefficient of determination to evaluate the model fit to the data; η_0 = single collector contact efficiency; α = sticking efficiency; λ = removal per meter distance, average removal from replicate experiments is shown.

silt, and clay (<32 μm; 4.7% in fine and 3.2% in medium MAR sand) reduce the pore space which can result in a retention of the bacteria by size-exclusion or a longer travel distance through the column. This increases contact time and the chance of attachment of bacteria to the sand material (Murphy and Ginn, 2000).

As observed in the quartz sand experiments, *E. coli* showed higher removal than the plant pathogenic bacteria comparing the BTCs where the peak C/C_0 is on average 2.3×10^{-3} for *E. coli* and 1.9×10^{-2} for *P. carotovorum*, and 1.4×10^{-2} for *R. solanacearum*. Moreover, k_{att1} coefficients were about ten times higher in medium aquifer sand ranging from 129 – 551 day^{-1}. Detachment at site 1 is negligible as values are small (<1 day^{-1}), similar to the quartz sand experiments. The highest removal was achieved in fine aquifer sand under anoxic and oxygenated conditions, where *P. carotovorum* had a k_{att1} of 765 day^{-1} and 515 day^{-1}, respectively. The peak C/C_0 values in fine aquifer sand were very low (1.0×10^{-4} under anoxic and 8.1×10^{-4} under oxygenated conditions) and effluent concentrations were at the limit of detection (Fig. 2C+D). As a result of the high removal, the breakthrough peak in fine sand columns was less pronounced in comparison to the shape of the BTCs in quartz and medium aquifer sand experiments. The effluent concentrations in the experiment with oxygenated fine sand were scattered and resulted in a poor model fit ($R^2 = 0.21$). Although the data of the anoxic fine sand experiments were also scattered, a reasonable fit using the 1-site kinetic model was achieved ($R^2 = 0.81$). Here, the initial breakthrough of the bacteria was noticeable which has a greater influence on R^2. Overall, all BTCs of *P. carotovorum* in aquifer sand were better fitted with the 1-site kinetic model, while the transport of *E. coli* and *R. solanacearum* in medium aquifer sand showed a better fit applying the 2-site kinetic model. The k_{att2} and k_{det2} values were in a similar range for *R. solanacearum* (about 25 day^{-1}), while for *E. coli*, k_{att2} was higher (75-132 day^{-1}) than k_{det2} (0.4-79 day^{-1}). The results agree with the observations by Schijven et al. (2002) that site 1 is characterized by relatively fast attachment and slow detachment, while site 2 is characterized by relatively fast attachment and fast detachment, implying that attachment to site 1 mostly determines removal.

3.4. Parameter evaluation

The 1-and 2-site kinetic models and associated parameter estimates resulted in a good fit of the bacterial breakthrough data as confirmed by AIC and R^2. Yet, we often found little differences between the 1- and 2-site kinetic models in terms of goodness of fit (AIC and R^2, Table S5), indicating both models would be a reasonable choice and that kinetic site 2 is less relevant (e.g. *E. coli* in quartz sand). The rate coefficients of kinetic site 2 influenced the shape of the BTC and improved model fit, as for example the BTC of *P. carotovorum* replicate 2 in anoxic medium aquifer sand (Fig. 2C). There, the 2-site kinetic model allowed a smooth transition from the declining limb towards the tail of the BTC, governed by k_{att2} and k_{det2} (Schijven et al., 2000). In contrast, the 1-site kinetic model (replicate 1 and 3) failed to model a smooth transition from the peak to the decline of the BTC and the curve shape of the tail. Nevertheless, Fig. 2C shows that the 1-site model represents sufficiently the curve shape of the BTCs. Consequently, site 2 was less important for the removal of the pathogens which was also represented by the high standard deviations of the parameters at site 2 (e.g. $k_{det2} = 49 \pm 39$ day^{-1}, *P. carotovorum* in quartz sand). The peak C/C_0 was mainly determined by k_{att1} which was confirmed by calculating C/C_0 using only k_{att1} as input value using Eq. (5). The calculated C/C_0 and modeled C/C_0 by Hydrus-1D were in the same range. k_{det1} determined the height of the tail and the values of C/C_0 at the tail are magnitudes lower than at the peak breakthrough in all experiments. In the aquifer sand experiments, the tail was about three orders of magnitude lower than the peak C/C_0 and in all experiment, the tail could only be visualized using the semi-log scale (see Fig. S3). Therefore, the removal mechanism can be simplified and only described by irreversible attachment that neglects detachment.

We compared results of the reversible attachment-detachment model with CFT to evaluate k_{att1}. In CFT, the removal of colloids (bacteria) is described with the single collector contact efficiency (η_0) and the sticking efficiency (α) from which an irreversible deposition rate can be calculated (Pang et al., 2021). The results of η_0 and α are shown in Table 2. η_0 was similar in quartz and aquifer sand (0.009-0.014). α values were low in quartz sand (0.018 – 0.176) reflecting little

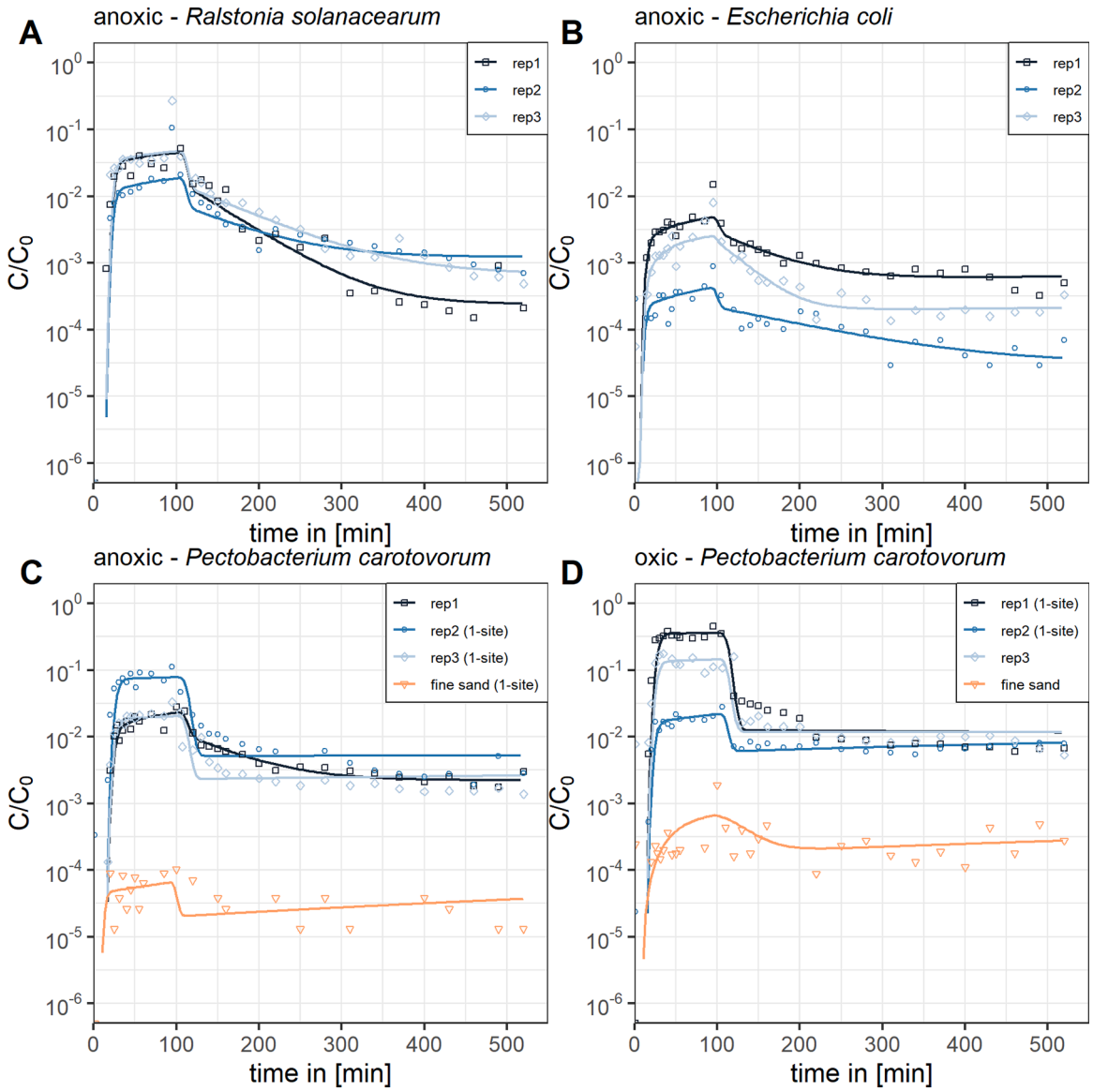

Fig. 2. Bacterial breakthrough curves in medium (shades of blue) and fine (orange) aquifer sand (rep = replicate). Normalized effluent concentrations (C/C_0) are plotted as a function of time (min). Solid lines represent the fitted models obtained from Hydrus-1D and the symbols represent the corresponding dataset in the same color. Two-site kinetic models are shown if not stated other in the legend. Triplicate experiments (rep. 1-3) in anoxic medium aquifer sand of *Ralstonia solanacearum* (A), *Escherichia coli* (B), and *Pectobacterium carotovorum* (C) are shown. Triplicate experiments in oxygenated medium aquifer sand of *P. carotovorum* are shown (D). BTC of *P. carotovorum* in fine aquifer sand is shown in orange under anoxic (C) and oxygenated (D) conditions.

attachment, while higher collision frequencies were represented by high α in aquifer sand (0.182 – 0.725). Previously reported (Hijnen et al., 2005) sticking efficiencies for *E. coli* WR1 in column experiments with finer natural sandy soil (d_{50} = 180 μm) were 0.341 at 0.5 m day^{-1} and 0.424 at 0.9 m day^{-1}, therefore lower than our results for *E. coli* in medium aquifer sand (α = 0.540 – 0.725, at 3.6 m day^{-1}). Moreover, CFT predicts that finer sand results in higher sticking efficiencies. Yet, in our results, sticking efficiencies of *P. carotovorum* in medium aquifer sand were similar or higher than in fine sand. CFT does not consider heterogeneities of the porous medium such as metal oxides that pose favorable attachment sites, nor physiological bacterial characteristics like hydrophobicity or surface charge. The attachment-detachment model accounts for these heterogeneities which is reflected in higher k_{att1} comparing transport of *P. carotovorum* in anoxic fine (765 day^{-1}) and medium aquifer sand (e.g. rep. 1: 184 day^{-1}). Finally, although k_{att1} mostly governs removal, detachment has to be taken into account as detached microorganisms re-contaminate the water source and might pose a risk to the end-user. Results of Knappett et al. (2014) pointed out detachment should not be ignored at the field scale.

3.5. Microbial removal rates

To evaluate our results on a larger scale we analyzed $\log_{10}$ removal per meter that only takes into account irreversible attachment to site 1. These ranged between 0.42 for *R. solanacearum* and 6.8 $\log_{10}$ m^{-1} for *E. coli* in quartz sand. A similar removal (4.69 log m^{-1}) in quartz sand columns (d_{50} = 250 μm) were obtained for *E. coli* J6-2 by Weaver et al. (2013). Our quartz sand experiments allowed to investigate bacterial transport under very controlled conditions as the sand has a defined grain size and no favorable attachment sites. There were no physicochemical differences between the quartz experiments, except that the experiment with *R. solanacearum* was done at room temperature. Previous research showed that temperature had little effect on bacterial deposition (Kim et al., 2009) but it did influence the retention of viruses (Sasidharan et al., 2017). Consequently, differences in transport are due to the nature of the bacterial species and their metabolic state which

influences cell characteristics like hydrophobicity and surface charge (Bradford et al., 2013; Murphy and Ginn, 2000).

Compared to quartz sand, the removal in anoxic medium aquifer sand was much higher for *R. solanacearum* (18 $\log_{10}$ m^{-1}) and *P. carotovorum* (19 $\log_{10}$ m^{-1}) and even 28 $\log_{10}$ m^{-1} for *E. coli*. Similar removal of about 20 log m^{-1} were presented by Oudega et al. (2021) using column experiments with natural heterogeneous gravel material and the bacterium *Bacillus subtilis* and a bacteriophage. Yet, the authors found a much lower removal in the field (0.2 log m^{-1}) which they explained by preferential flow paths that may occur in their studied aquifer and enhance the bacterial transport. These large discrepancies between column and field studies have also been summarized by Pang (2009) and need to be considered in risk assessments. The highest removal (40 $\log_{10}$ m^{-1}) was observed in oxic fine aquifer sand for *P. carotovorum*. This highlights the importance to know about the geological structure of a site. Although the aquifer sands used in our study had a similar d_{50} of 192 μm (fine) and 305 μm (medium), their removal capacity differed greatly. This may be caused, amongst others, by the very fine fraction (<125 μm) which was 10% in fine sand and only 3% in medium aquifer sand (Table S1). Therefore, a detailed geological description of a MAR site is very useful to predict removal of pathogens and field tests should be included if feasible.

3.6. Influence of oxygenation

In this research, the influence of oxygenation of the aquifer sand material and effects on the transport of *P. carotovorum* was investigated. The column filled with medium aquifer sand was first operated under anoxic conditions followed by flushing the column with oxic aGW to simulate infiltration of oxic water into an anoxic aquifer. Oxygen levels in the column influent and effluent remained below 0.1 mg L^{-1} during anoxic experiments. During the flushing, the influent O_2 concentration was about 12 mg L^{-1}, while the effluent O_2-levels were on average 1.4 mg L^{-1} lower than the influent indicating O_2 consumption by chemical and/or biological reactions within the column. Reactive transport modelling has shown that the injection of oxic water into an anoxic aquifer will modify the biological and geochemical properties of the aquifer and thereby, could affect the microbial transport over time (Kruisdijk and van Breukelen, 2021). Ferrous iron (e.g. from minerals like pyrite) is oxidized and results in formation of positively charged iron oxide coatings which can increase bacterial attachment due to their negative surface charge (Johnson et al., 1996). For example, virus attachment was reduced under anoxic conditions in field experiments (Schijven and Hassanizadeh, 2000), while sub(oxic) conditions increased virus removal due to positively charged metal oxides (Hornstra et al., 2018). In contrast, our results unexpectedly show that removal of *P. carotovorum* decreased under oxygenated conditions compared to prior anoxic conditions. The average removal was 19 (± 1.3) $\log_{10}$ m^{-1} under anoxic and 13 (± 4) $\log_{10}$ m^{-1} under oxic conditions. The transport in oxygenated fine aquifer sand also resulted in lower removal, although the flushing with oxygen-rich water was done for a shorter time (2 days). Here, the removal was 40 $\log_{10}$ m^{-1} under anoxic and 31 $\log_{10}$ m^{-1} under oxic conditions. On the one hand, lower retention during oxic experiments may have been a result of fewer available attachment sites due to retained cells of *P. carotovorum* during the anoxic medium MAR experiments. On the other hand, we expect that most the bacteria died-off during the two weeks flushing with oxic aGW (Eisfeld et al., 2021) or detached during flushing with deionized water which changed the ionic strength (Foppen et al., 2007). Moreover, the retention of *P. carotovorum* in oxic medium MAR sand in replicate 2 was just as high (k_{att1} = 215 day^{-1}) as during the anoxic experiments (k_{att1} = 129-202 day^{-1}) which indicates that there were still sufficient attachment sites available. The two other replicate experiments (1+3) under oxic conditions showed less removal under oxic conditions calculated sticking efficiencies that were lower in oxic compared to the anoxic experiments in medium and fine aquifer sand.

As stated earlier, oxygen-rich water modifies the geochemistry of an anoxic aquifer, but also acts as an environmental signal for microorganisms that may trigger metabolic changes which consequently modifies attachment. As response to anoxic conditions, microorganism can modify their gene expression which may lead to the production of different surface structures (e.g. lipopolysaccharides) and alterations in surface charge as shown for *E. coli* (Landini and Zehnder, 2002). Castro and Tufenkji (2008) analyzed the transport of *E. coli* O157:H7 and *Yersinia enterocolitica* under low and high oxygen concentrations. While *E. coli* had a higher sticking efficiency under oxic conditions, attachment of *Y. enterocolitica* was increased under anoxic conditions. The authors assumed that growing the bacteria either aerobically or anaerobically resulted in differences in the cell surface structure and surface charge. Similarly, the plant pathogenic bacteria have adaptation strategies that changes their transcriptional response and surface properties in response to environmental changes (Lisicka et al., 2018). To conclude, expected improvement in removal due to oxygenation was not observed as the duration of the oxygenation may have been insufficient to increase the presence of iron-oxides. Future research is needed to further investigate the effects of oxygenation on the porous media and its effects on the pathogens and natural microbiota to evaluate it overall effects on pathogen transport.

3.7. Applicability to MAR systems

Column experiments are an important tool in the assessment of bacterial transport because environmental conditions can be better controlled than in the field. Nevertheless, upscaling of these results to field scale needs to be done with caution as results may overestimate the actual removal in the field, particularly when the variation in grain size distribution with depth is poorly characterized (Oudega et al., 2021; Pang, 2009). The quality of the water used for injection should also be considered in translating column results to the field scale. In our study, high inoculation concentrations (10^6 CFU mL^{-1}) were used to enable measuring the breakthrough over a longer period, but this does not represent concentrations of plant pathogenic bacteria found in surface waters which are about 0.1 - 10^3 CFU mL^{-1} (Wenneker et al., 1999). Moreover, the applied flow velocity of 3.6 m day^{-1} represents high groundwater flow during constant injection. Under realistic conditions, water will be injected during rain events but recovered at a different time point and the velocity in the aquifer will be lower especially during periods without infiltration. Lower velocity results in higher contact time of the pathogens with the grain surface which favors attachment, and residence time is an essential factor in MAR operation (Hendry et al., 1999; Massmann et al., 2008). Field experiments with pathogenic organisms are often prohibited. Therefore, we included *E. coli* WR1 as reference organism for future field experiments. Yet, *E. coli* WR1 had the highest removal in quartz and aquifer sand in comparison to the plant pathogenic bacteria which makes it unsuitable for direct comparison to these pathogens. In a future study, an alternative surrogate for field experiments should be tested in column experiments beforehand to evaluate the comparability with the plant pathogens. Nevertheless, the results showed that the aquifer sands were an effective filter to remove bacterial pathogens. Future work will combine the results of this study on filtration processes with those of die-off experiments with the same plant pathogens (Eisfeld et al., 2021) in a quantitative microbial risk assessment model to aid the design (e.g. minimal travel distances and residence times) of MAR systems.

4. Conclusions

Plant pathogenic bacteria are a threat to global agricultural production and dissemination of plant diseases via contaminated irrigation water has often been observed. Irrigation water availability is under pressure due to water scarcity and the deterioration of (surface) water quality. We studied if aquifer storage and recovery as solution for water

scarcity could deliver safe irrigation water by means of plant pathogen removal during transport in natural sediments. In laboratory column experiments, we showed the effective removal of three plant pathogenic bacteria in natural sediments from a MAR site and compared the transport with the human pathogen *E. coli* WR1. Removal parameters were obtained by modeling the results in Hydrus-1D. The heterogeneous natural aquifer sands had a higher (up to several $\log_{10}$) pathogen removal capacity than quartz sand. Oxygenation of the anoxic aquifer sands did not increase removal, contrary to our expectations. Tailing caused by detachment has been observed in all BTCs but was several magnitudes lower than the peak C/C_0 suggesting that it may be neglected. However, detachment has to be considered as trace levels of highly infectious pathogens may still be present after soil treatment. The quartz sand experiments represent non-favorable conditions for attachment and results may be used as worst-case scenario in risk assessments. In conclusion, our study confirms the potential of MAR as a natural water treatment technology in agricultural settings to store and supply both fresh, and bacterially safe irrigation water.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

The supporting information consists of a total of 14 pages with seven tables and three figures. S1 describes details on porous media and aqueous solutions preparation and S2 contains a detailed characterization of bacteria and culture media. Table S1 shows the grain size analysis and Table S2 the chemical analysis of the aquifer sands used in the column experiments. Table S3 shows the fitting parameters of the tracer BTCs and Table S4 the results of the hydrophobicity (MATH) test. Table S5 lists the R^2 and AIC output parameters from Hydrus-1D to compare the 1-and 2-site kinetic model. Table S6 shows results of the iron content of the sands used in the experiments. Fig. S1 shows the grain size distribution chart of the used sands. Fig. S2 shows the experimental column setup in the laboratory and Fig. S3 shows the BTCs in quartz sand on a linear an semi-logarithmic scale.

Declaration of Competing Interest

The authors declare no competing interests.

Funding sources

This research has been financially supported by the Netherlands Organization for Scientific Research (NWO; Topsector Water Call 2016; project acronym AGRIMAR; contract number: ALWTW.2016.023; https://www.nwo.nl/onderzoeksprogrammas/topsector-water-call) with co-funding from private partners Acacia Water B.V. (acaciawater.com), Broere Beregening B.V. (broereberegening.nl), and Delphy B.V. (delphy.nl). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The funders did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgment

We like to thank the Department Ecology and Biodiversity at the University of

Utrecht to facilitate the work with *R. solanacearum* in the quarantine laboratory. We also thank the anonymous reviewers for their constructive comments to improve our manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118724.

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