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Transport of bacteriophage MS2 and PRD1 in saturated dune sand under suboxic conditions

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ABSTRACT

Soil passage of (pretreated) surface water to remove pathogenic microorganisms is a highly efficient process under oxic conditions, reducing microorganism concentrations about 8 $\log^{10}$ within tens of meters. However, under anoxic conditions, it has been shown that removal of microorganisms can be limited very much. Setback distances for adequate protection of natural groundwater may, therefore, be too short if anoxic conditions apply. Because removal of microorganisms under suboxic conditions is unknown, this research investigated removal of bacteriophage MS2 and PRD1 by soil passage under suboxic conditions at field scale. At the field location (dune area), one injection well and six monitoring wells were installed at different depths along three suboxic flow lines, where oxygen concentrations ranged from 0.4 to 1.7 mg/l and nitrate concentrations ranged from 13 to 16 mg/L. PRD1 and MS2 were injected directly at the corresponding depths and their removal in each flow line was determined. The highest bacteriophage removal was observed in the top layer, with about 9 log removal of MS2, and 7 log removal of PRD1 after 16 meters of aquifer transport. Less removal was observed at 12 m below surface, probably due to a higher groundwater velocity in this coarser grained layer. MS2 was removed more effectively than PRD1 under all conditions. Due to short travel times, inactivation of the phages was limited and the reported log removal was mainly associated with attachment of phages to the aquifer matrix. This study shows that attachment of MS2 and PRD1 is similar for oxic and suboxic sandy aquifers, and, therefore, setback distances used for sandy aquifers under oxic and suboxic conditions provide a similar level of safety. Sticking efficiency and the attachment rate coefficient, as measures for virus attachment, were evaluated as a function of the physico-chemical conditions.

1. Introduction

Soil passage of (pretreated) surface water to remove pathogenic microorganisms is frequently applied in drinking water production, and takes place during artificial recharge of groundwater aquifers (Bouwer, 2002), riverbank filtration (Stuyfzand, 1989a; Tufenkji et al., 2002), and, specifically in the Netherlands, in dune infiltration (Stuyfzand, 1989b, 1999). During soil passage pathogenic microorganisms are removed by attachment of the microorganisms to soil particles and by inactivation or die-off (Bradford et al., 2014; Schijven and Hassanizadeh, 2000). The combination of virus persistence in the aquatic environment, their ease of being transported with the water and through soil and filter media and their infectivity, make viruses a significant microbial hazard for drinking water safety (Schijven and Hassanizadeh, 2000).

Bacteriophages MS2 and PRD1 have been used extensively as model viruses in field and laboratory studies (Schijven and Hassanizadeh, 2000). Bacteriophage MS2 is a group I F-specific bacteriophage. It is icosahedral with a diameter of 26 nm and has a low isoelectric point of 3.5, implying it has a negative surface charge under most conditions. As Schijven and Hassanizadeh

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(2000) have reviewed, in most soils it therefore attaches less than or as poor as other negatively charged viruses. For example, a coxsackievirus B4 attaches as poor as MS2, whereas, the less negatively charged, poliovirus 1 attaches much more (Schijven et al., 2003). At low temperatures, it is relatively persistent, but much less so at temperatures above 10 °C. Harvey and Ryan (2004) and Schijven and Hassanizadeh (2000) reviewed literature on the use of bacteriophage PRD1 as a model virus. It is an icosahedral bacteriophage with a diameter of 62 nm. It attaches about as poorly as MS2. It has the advantage over MS2 that it is much more persistent, in fact more than most viruses (Bertrand et al., 2012). The combination of these two properties make it an excellent precautions model virus. PRD1 has structural and functional similarities with mammalian adenoviruses.

In field studies on oxic dune infiltration (Schijven et al., 1999) and anoxic deep well injection with water containing oxygen (Schijven et al., 2000) in sandy aquifers, it was demonstrated that removal of bacteriophages MS2 and PRD1 as model viruses was very effective. About 8 $\log_{10}$ removal was achieved after 30 m and 38 m of soil passage, respectively. These studies also showed that virus removal was nonlinear with travel distance, *i.e.* most of the removal took place during the first tens of metres of soil passage. Thereof, removal was less efficient in the deep well injection study. This nonlinear trend in removal with travel distance was ascribed to the presence of ferric oxyhydroxides in the oxic zone over the first meters of soil passage that provided favourable sites for virus attachment and absence of these attachment sites in the anoxic zone that entails the residual travel distance (Schijven et al., 2000).

Schijven et al. (2006, 2010) used the virus attachment and inactivation data from those field sites (Schijven et al., 1999, 2000) to calculate setback distances for protection of natural groundwater against virus contamination from leaking sewers in order to comply with a maximum infection risk of one per ten thousand persons per year. Virus removal by soil passage, and hence, setback distance, is highly sensitive to the values of virus attachment and inactivation (Schijven et al., 2006). If geochemical conditions at a natural groundwater abstraction site are unknown, it is suggested to apply the conservative value for virus attachment. The conservative value for virus attachment in a sandy aquifer originates from measurements in the anoxic zone of the deep well injection site (Schijven et al., 2000); and expressed as a sticking efficiency (Yao et al., 1971), it was in the order of 10^{-5}. This low sticking efficiency was confirmed in another field study under anoxic conditions, where in anoxic sandy soil, only a little more than 2 log removal of MS2 was observed after 30 meters of soil passage (Van der Wielen et al., 2008). Note that favourable sites for attachment in the form of metal oxyhydroxides are absent under anoxic conditions. In addition, favourable sites for attachment may be absent, or low, when they are blocked, *e.g.* by organic matter (Pieper et al., 1997; Ryan et al., 1999), or at high pH and/or low ionic strength (Schijven and Hassanizadeh, 2000). The required setback distances under anoxic conditions were calculated to correspond to travel times of 110 days to one year, instead of the currently applied 60 days. If it can be demonstrated that when conditions in an aquifer are less unfavourable for virus removal than abovementioned, a smaller setback distance may be sufficient to produce safe drinking water from groundwater (Schijven et al., 2006).

In the Netherlands, redox conditions in abstracted groundwater vary from (sub)oxic to deeply anoxic, or constitute a mixture of these conditions. Most groundwater is anoxic ($O_2 < 0.5$ mg/l, NO_3^- <0.5 mg/l and Mn^{2+} and $Fe^{2+} \geq 0.1$ mg/l) to deeply anoxic (as anoxic but with significant sulphate reduction or methane production). In the central and eastern part of the Netherlands, with most of the phreatic sandy aquifers, about one fifth of the abstracted groundwater is suboxic (1 mg/l < O_2 < 80% saturation and $NO_3^- \geq 1$ mg/l). Oxic water is characterised by ≥ 1 mg/l O_2 and ≥ 1 mg/l NO_3^-. Table 1 gives some exploitation data for the four major groundwater production areas in the Netherlands in 2008 (Mendizabal and Stuyfzand, 2009).

The field studies of Schijven et al. (1999, 2000) and Van der Wielen et al. (2008) provide estimates for virus attachment in sandy aquifers under oxic and anoxic conditions. Under oxic conditions, attachment conditions is favourable and, therefore, virus removal is efficient, whereas under anoxic conditions, virus attachment is poor with considerable implications for the size of groundwater protection zones. This finding was supported by column studies under oxic and anoxic conditions, where, in line with the field studies, less adsorption was observed under anoxic conditions (Frohnert et al., 2014). These studies show that redox conditions play an important role in attachment of viruses, but has been investigated so far under only under oxic and anoxic conditions. Suboxic conditions are common, but the efficiency of virus removal under these conditions is unknown. Therefore, the current study aimed to fill this knowledge gap by investigating virus attachment under suboxic conditions at field scale. To that aim, transport of bacteriophages PRD1 and MS2 as model viruses was studied at field scale in a dune area with artificial recharge of groundwater under suboxic conditions in a sandy aquifer.

2. Materials and methods

2.1. Site description

The field study was performed in the Amsterdam Water Supply Dunes, at about 15 km southwest of Amsterdam. The area is in use by drinking water company Waternet for infiltration of pretreated river water to be recovered and post-treated to drinking water quality (61 Mm^3/y). The water infiltrates in 40 infiltration ponds and is, after soil passage, collected in drains and open canals from where it is transported to the drinking water production plant for further treatment. The experimental site was located between infiltration pond 6 and a collection canal. The horizontal distance between infiltration pond 6 and the drainage canal is 101 m. The water level of the infiltration pond is about 5.2 m above the level of the canal. The groundwater table is about 2 m below soil surface, and groundwater with low oxygen levels were found between 10 m and 12 m below soil surface. Soil surface and water tables are related to NAP (Normal Amsterdams Peil, Normal Amsterdam level, a zero reference level). At this location, 10 meter below surface relates to NAP -6.5 m.

2.2. The aquifer and its monitoring wells

The aquifer consists of sand (97.6%) and 2.4% of clay and silt. There are occasional layers of intact shells at various depths. Around monitoring well PL3, nearby filter screens 2 and 3, the soil contained with 93.4% sand, 3.3% silt and 3.1% clay more clay and silt

Table 1
Average exploitation data for four hydrosomes (groundwater areas) in the Netherlands in 2008 (Mendizabal and Stuyfzand, 2009).

Hydrosome	Central	East	North	South
Number of public supply well fields	52	31	27	42
Mm^3/year	168	8	126	105
% of total drinking water production	18	9	14	11
Suboxic	21	19	0	0
Anoxic	40	55	56	83
Deeply anoxic	25	6	37	10
Mixed	13	19	7	2

than measured around the other monitoring well, suggesting local heterogeneity nearby PL3. The porosity of the aquifer is 0.38. The median grain size varies between 0.24 and 0.30 mm. The pH of the groundwater was 7.9 and water temperature during injection of the bacteriophages was 15.2 °C. The soil organic matter (SOM) varied between 0.24 and 0.47%. Cation exchange capacity varied between 8 and 23 meq kg^{-1}. Geochemical analysis of the soil is described in Table 3. A description of the drillhole columns is added in Fig. 1.

Six monitoring wells (PL2–PL7) were installed in line with the groundwater flow direction at 2.5 m, 5 m, 10 m, 18 m, 28 m and 32 m distance from the injection well (PL1) with filters screens of 0,25 m length at 6.5, 7.5 and 8.5 m below NAP (Fig. 2). Outside the central line of supposed groundwater flow, four additional monitoring wells (PD9–PD11) were installed for monitoring bacteriophages that could follow flow paths slightly deviating from the central line. The injection filter screen was 3 m in length with a diameter of 28 cm and was divided into three sections, each 1 m in length. The depth of each section corresponded with the depths of the screens of the monitoring wells (Table 2). The reason for injection and monitoring at three different depths was to encompass the desired suboxic conditions, which could not be predicted exactly before the installation of the wells. Suboxic conditions were determined based on the oxygen levels in the groundwater of the injection well and the monitoring wells prior to the start of the experiment. With a concentration of respectively 1.7, 0,7 and 0.8 mg/l O_2 at 6.5, 7.5 and 8.5 m below NAP in the injection well, suboxic conditions were confirmed at 7.5 and 8.5 m below NAP, while at 6.5 m below NAP the conditions where close to suboxic. Downstream of the injection well, in PL2 to PL6 the oxygen concentration remained suboxic, with 0,7 and 0.8 mg/l at 7.5 and 8.5 m below NAP. At 6.5 m below NAP the oxygen concentration downstream of the injection well gradually decreased to suboxic, with a concentration of 0,5 mg/l oxygen in monitoring well PL6.

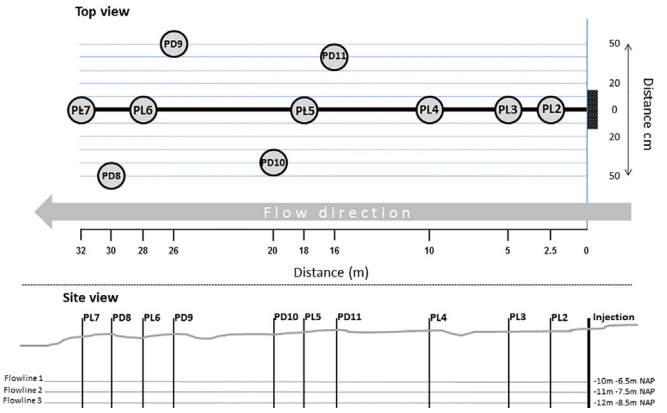

Fig. 2. A graphical overview of the experimental set up showing the position of the injection well and the monitoring wells. PL2 to PL7 are positioned in line with the flow path, while PD8 to PD11 are located just outside the flow path.

2.3. Salt tracing

Salt tracing was conducted in order to estimate interstitial flow velocity and medium dispersivity. In relatively homogeneous sandy aquifers, the flow velocity of a salt tracer and its dispersivity are considered to be representative for virus particles (Schijven et al., 1999, 2000, Van der Wielen et al., 2008). Each injection filter section was connected to a separate 1000-L water tank that was filled with water taken from the corresponding aquifer depth. Oxygen levels in the tanks were kept as constant as possible by means of a constant flow of nitrogen gas. Initial oxygen levels of 1.9, 0.9 and 0.8 mg/l in tanks 1, 2 and 3, respectively, dropped to 0.8, 0.4 and 0.4 mg/l after 48 h of injecting. A solution of 0.9 g/l sodium chloride

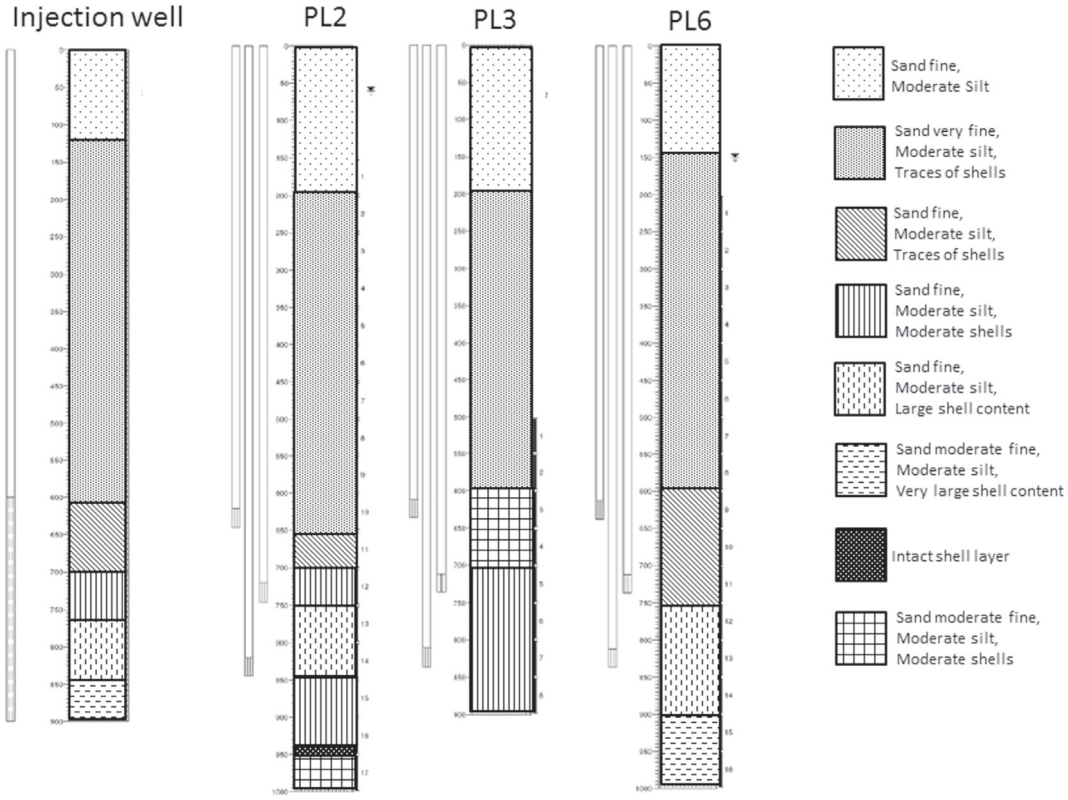

Fig. 1. The soil characteristics are reflected in the drill hole columns, from the injection well, and monitoring well PL2, PL3 and PL6.

Table 2
Position of wells, pore water velocities, dispersivities, initial concentrations and dilution factor of bacteriophages MS2 and PRD1.

Well[a]	Distance from injection point (m)	Depth NAP (m)	v (m/d)	α_L (m)	$C_{0,MS2}$ (pfu/ml)	$C_{0,PRD1}$ (pfu/ml)	Dilution
PL2-1	2.5	−6,5	1.1	0.048	3,1E^8	1,7E^7	No
PL3-1	5	−6,5	1.1	0.011			
PL4-1	10	−6,5	1.1	0.010			
PD11-1	16	−6,5	1.1	0.21			
PL2-2	2.5	−7,5	1.1	0.12	3,1E^8	1,7E^7	No
PL3-2	5	−7,5	1.1	0.048			
PL4-2	10	−7,5	1.1	0.087			
PD11-2	16	−7,5	1.1	1.8			
PL2-3	2.5	−8,5	2.1	0.49	2,4E^8	1,3E^7	0.77
PL3-3	5	−8,5	2.1	0.40			
PL4-3	10	−8,5	2.1	0.10			
PD11-3	16	−8,5	2.1	4.5			
PL5-3	18	−8,5	2.1	0.42			

[a] The last digit (1, 2 or 3) refers to upper (1), middle (2) or lower (3) flow path.

was injected during 48 h from the water tanks with a flow rate of 12.9 l/h. The transport of the sodium chloride was monitored in 15-min intervals by measuring electric conductivity (EC) with calibrated CTD-divers in the monitoring wells. The EC data were used to construct salt tracer breakthrough curves.

2.4. Bacteriophages preparation, injection and monitoring

A high-titer bacteriophage suspension of MS2 was obtained from GAP Environmental Services (London, Canada) at a concentration of 9.1×10^{10} plaque forming units (pfu)/ml. Bacteriophage PRD1 and host strain *S. typhimurium* LT2 were obtained from the National Institute of Public Health and the Environment, Bilthoven, the Netherlands (RIVM). A high-titer suspension of 5.4×10^9 pfu/ml of PRD1 was prepared according to ISO10705-1 (1995). Initial concentration of the bacteriophages in the injection tanks was aimed at about 10^8 and 10^7 pfu/ml, for MS2 and PRD1 respectively. Of each tank, daily samples were taken to determine initial concentrations of the bacteriophages. Both bacteriophages were injected simultaneously at a flow rate of 12.9 l/h for 64 h.

Each of the 3 filter screens of the monitoring wells was equipped with a tube for sampling. The other end of each tube was placed at about 75 m downstream the experimental area to avoid cross contamination. This area was 4 m lower than the monitoring wells, therefore, the tubes could take water from the screens by using the natural gradient as driving force. The end of each tube was connected to a metal sampling tap that was flame-sterilized before taking a sample. Each sample was taken at a rate of 1 l/min, whereby the first 6 l (about three times the tube volume) were discarded to obtain a representative sample from the well. The monitoring wells were sampled for MS2 and PRD1 at regular intervals up to forty days after injection. Bacteriophages MS2 and PRD1 were enumerated as described in ISO 10705-1 (1995) using *S. typhimurium* WG49 as the host for MS2 and *S. typhimurium* LT2 as the host for PRD1. Nalidixic acid was omitted for PRD1.

2.5. Inactivation of MS2 and PRD1 in groundwater

Inactivation of free MS2 and PRD1 was determined in groundwater from the experimental site. To that aim, samples were collected from the three screens of monitoring well 2 after 2,5 m of soil passage, when peak breakthrough concentrations were expected. The samples were collected in eight bottles per screen depth that were filled completely, and stored in the lab at the corresponding groundwater temperature of 15 °C. During 70 days, a bottle per screen depth was taken at regular intervals to enumerate MS2 and PRD1 bacteriophages as described above.

2.6. Conceptual model

The major processes included in the mathematical model of our field study are advection, hydrodynamic dispersion, attachment, detachment and inactivation. Given the hydrodynamics of the experimental site and the placement of the filter screens in the monitoring wells, the flow of water from well to well was assumed to be one-dimensional.

The governing equations for an advection–dispersion model, including reversible adsorption to two types of kinetic sites 1 and 2, and inactivation of free and attached bacteriophages, in the case of uniform one-dimensional flow, are as follows (Schijven et al., 2002), and including blocking of sites 1 and 2 (Sasidharan et al., 2014):

$$\frac{\partial C}{\partial t} + \frac{\rho_B}{n}\left(\frac{\partial S_1}{\partial t} + \frac{\partial S_2}{\partial t}\right) = \frac{\partial}{\partial x}\left(\alpha_L v \frac{\partial C}{\partial x}\right) - v\frac{\partial C}{\partial x} - \mu_l C - \frac{\rho_B}{n}\mu_{s1} S_1 \quad (1)$$

$$\frac{\rho_B}{n}\frac{\partial S_1}{\partial t} = \left(1 - \frac{S_1}{S_{\max 1}}\right) k_{a1} C - \frac{\rho_B}{n}\left(k_{d1} S_1 + \mu_{s1} S_1\right) \quad (2)$$

$$\frac{\rho_B}{n}\frac{\partial S_2}{\partial t} = \left(1 - \frac{S_2}{S_{\max 2}}\right) k_{a2} C - \frac{\rho_B}{n} k_{d2} S_2 \quad (3)$$

Subject to boundary conditions $C = C_0$ at $x = 0$ and $\frac{\partial C}{\partial x} = 0$ at $x = L$, where L is the transport length. C is the concentration of free bacteriophages (pfu/ml); S is the concentration of attached bacteriophages (pfu/g); t is the time (days); x is the travel distance (m); α_L is the longitudinal dispersivity (m); v is the mean interstitial water velocity (m/day); ρ_B is the dry bulk density (kg/m^3); n is the porosity; k_a and k_d are the attachment and detachment rate coefficients, respectively (day^{-1}); μ_l and μ_s are the inactivation rate coefficients of free and attached bacteriophages, respectively (day^{-1}). Subscripts 1 and 2 refer to the two different kinetic sites. $S_{\max}$ is the maximum solid phase concentration of attached bacteriophages to site 1. When the value of $S_{\max}$ is large, the blocking function (equations (2) and (3)) approaches the value of 1 and time-dependent deposition behaviour becomes irrelevant. Sites of type 2 are characterised by fast attachment and fast detachment, for that reason, inactivation of attached bacteriophages to site 2 does not need to be considered (Schijven et al., 2002).

2.7. Parameter estimation

Porewater velocities (v) and dispersity values (α_L) of were estimated by fitting the breakthrough curves (BTCs) from the salt tracing using Hydrus-1D version 4.16.0110 (Simunek et al., 2005). The duration of the salt tracer injection and the salt concentration at the injection point were used as boundary condition for the BTCs at the three depths of the first well (PL2-1, PL2-2 and PL2-3). For the next wells, the breakthrough concentration data of the upgradient wells at the corresponding depths were taken as boundary condition. The reason for this well-to-well approach is explained below with the fitting of the virus BTCs.

The inactivation rate coefficients (μ_l) of the bacteriophages in the water phase were estimated by linear regression of the time series of the log concentration of the bacteriophages from the groundwater collected from well 2. The slopes of tails of the breakthrough curves (BTCs) for the bacteriophages represent the inactivation rate coefficients (μ_s) of the attached bacteriophages

(Schijven et al., 1999). These inactivation rate coefficients were estimated for each bacteriophage for all BTCs, also by linear regression analysis. Whether μ_s values differ or did not differ between BTCs was determined for each bacteriophage by likelihood ratio testing (Cox and Hinkley, 1974).

Using Hydrus-1D, attachment and detachment rate coefficients were estimated by fitting the BTCs of the logarithmic virus concentrations to the two-site kinetic model with and without blocking as well as to the one-site kinetic model with and without blocking. In addition, assuming that favourable attachment is irreversible and unfavourable attachment is reversible, also the models with reversible attachment to site 1 and irreversible attachment to site 2 with and without blocking were tested (Sasidharan et al., 2014).

Commonly, logarithmic transformation of the BTC concentrations provide a better fit of the tail of the BTC (Schijven et al., 2002). Like for the salt tracer, duration and concentration of the bacteriophages at the injection point were used as boundary conditions for the BTCs at the three depths of the first well (PL21, PL22 and PL23). For the following wells, the breakthrough concentration data of the previous wells at the corresponding depths were taken as boundary condition. The estimates of the attachment and detachment rate coefficients from well to well may be correlated with hydrochemical conditions at the respective monitoring wells.

To investigate whether blocking played a role and whether two-site kinetics were required to describe the breakthrough curves adequately, the Akaike's Information Criterion (AIC) was used. Hydrus-1D provides values of AIC that make model selection possible. Given the availability of data that make up the BTC, a model with less parameters, like one-site kinetic and no blocking, may be sufficient to describe the BTC. In this regard, the model with the lowest AIC value was chosen as the best model. In the case of equal AIC values, the model with the least number of parameters (the simpler model) was chosen.

Colloid filtration theory (Tufenkji and Elimelech, 2004) was used to express virus attachment to site 1 in terms of sticking efficiency:

$$\alpha = \frac{2}{3}\frac{d_c}{(1-n)}\frac{k_{a1}}{v}\frac{1}{\eta_0} \quad (4)$$

where α is the sticking efficiency, d_c is the grain size diameter, n is the porosity and η_0 is the single collector contact efficiency.

2.8. Regression analysis

In order to investigate the dependence of the sticking efficiency on the physico-chemical conditions, multivariate regression analysis in combination with best model selection was conducted with the linear model function *lm* and the step function of the statistical package R (version 3.2.2), in which terms were removed based on AIC comparison, with parameter k = 3.84 (Venables and Ripley, 2002). This means that selected models with one parameter (degree of freedom) difference are compared by their likelihood values on a 5% (Chi-square distribution) basis. A logit transformation of the response variable (sticking efficiency α) was applied because α needs to stay between 0 and 1: logit(α) = log (α/(1-α)).

3. Results

3.1. NaCl as conservative tracer and parameter estimation

Before the injection of the bacteriophages, NaCl was injected as conservative tracer to confirm the flow direction of the water, and determine the residence time from the injection well to the monitoring wells. Furthermore, the breakthrough curves of NaCl, obtained from the monitoring wells were used to determine interstitial flow velocity, dispersivity and dilution from the injection well to the monitoring well. Fitting of the NaCl breakthrough curves using Hydrus-1D version 4.16.0110 resulted in values for pore water velocity and dispersivity (Table 2). Fig. 3 depicts the fitted salt tracer breakthrough curves. The curves are fitted generally well, except the irregularities of the climbing limbs at PL2-2 and PL2-3 could not be captured. Probably some physical heterogeneity plays a role here. The dispersivity values listed in Table for PD11-1, PD11-2 and PL5-3 were taken from fitting the

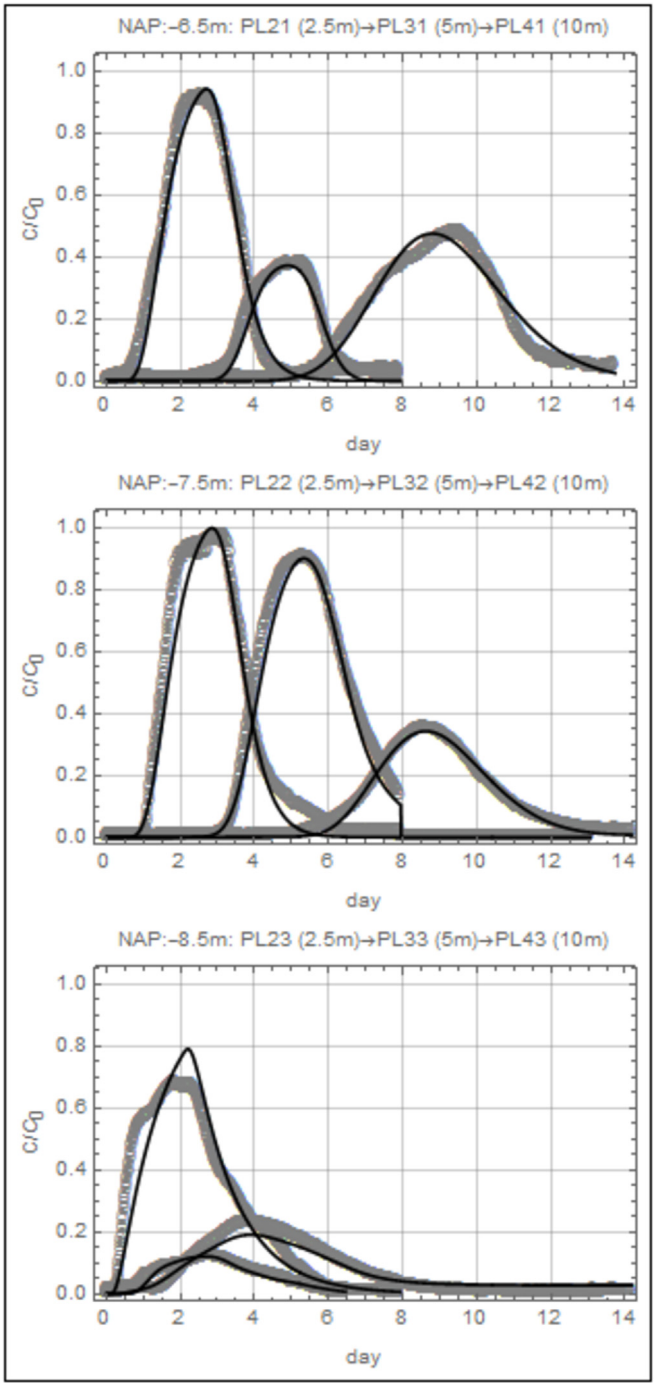

Fig. 3. Breakthrough curves of the salt tracer. Symbols are measurements of normalised electric conductivity, the headings indicate the wells that were monitored.

bacteriophage breakthrough curves, using the flow velocities from the salt tracer at the corresponding depths. Average porewater velocity was estimated to be 1.1 m/d at depths of NAP -6.5 and −7.5 m, and 2.1 m/d at NAP -8.5 m. Apparently, at NAP -8.5 m, hydraulic conductivity is about twice as high. Derived dispersivities were generally small, but larger at NAP -8.5 m. The combination of layers with higher clay and silt contents (Table 3) and layers of intact shells with higher hydraulic conductivity suggests the presence of preferential flow patterns at this depth.

3.2. Description of breakthrough of bacteriophage MS2 and PRD1

Initial concentrations of the bacteriophages at the injection well are included in Table 2. These concentrations were constant during the 64-h injection. For presentation of the bacteriophage BTCs, all bacteriophage breakthrough concentrations were normalised with the initial concentrations at the injection well. Due to the higher groundwater flow velocity at NAP -8.5 m, the bacteriophages injected at this depth were diluted by groundwater after injection (Table 2). All BTCs are characterised by a climbing limb, a peak concentration, a declining limb, and finally a gradually declining tail (Fig. 4). Judged from the lack of a maximum concentration plateau, a steady state condition was not achieved. The normalised peak concentration values $log_{10}(C_{max}/C_0)$ provide, therefore, an indication of the log removal of the bacteriophages. Fig. 5 presents $log_{10}(C_{max}/C_0)$ values corrected for dilution, of the bacteriophages versus travel distance, for the two bacteriophages, and for the three depths along which bacteriophage breakthrough was monitored. The oxygen concentration in the upper layer (NAP -6.5 m) was between 1.1 and 1.7 mg/l O_2, while oxygen concentration in the middle and lower layer was below 1 mg/l O_2. The removal of both phages was not identical in the upper and middle layer, and the difference in oxygen concentration might play a role, but physical heterogeneity in the aquifers soil is expected to be a larger factor affecting removal than the small oxygen concentration difference between these layers. Specifically around PL3, located 5 meter downstream of the injection point the soil samples showed a deviating composition when compared to the other soil samples (Table 3 and Fig. 1), and this is reflected in the large difference in removal efficiencies after 5 meters of soil passage. The pore water velocity was, with 2.1 m/d at NAP -8.5, substantially higher than the pore water velocity of 1.1 m/d at NAP -7.5 and −6.5 m. A higher pore water velocity was associated with less removal at all sampling points and for both phages. Furthermore, PRD1 removal is less than MS2 removal. Over 16 m and depending on the depth, PRD1 is removed 4–7.2 log_{10}, and MS2 is removed 8.5–9.1 log_{10}, which is highly efficient. In PL5, 18 meters downstream of the injection, PRD1 was found only in the layer at NAP -8.5 m. In the additional three monitoring wells beyond PL5 (more than 18 m), no bacteriophages were detected.

3.3. Inactivation of bacteriophage MS2 and PRD1 in groundwater

To determine the inactivation of MS2 and PRD1, samples were collected after 2.5 m of soil passage from the three different filter screens. Due to removal in the first 2.5 meters, the initial concentration was in the range of 9.4×10^5–3.5×10^7 PFU/ml for MS2 and 3.0×10^5–1.6×10^6 PFU/ml for PRD1. Inactivation of the bacteriophages was monitored for 70 days (Fig. 6). The inactivation rate coefficients of MS2 and PRD1 (μ_l), assuming first order inactivation, were on average log_{10} 0.14 day^{-1} and 0.06 day^{-1}, respectively.

3.4. Inactivation of bacteriophage MS2 and PRD1 attached to soil

From likelihood ratio testing, the first-order inactivation rate coefficient for the solid phase (μ_s) of PDR1 was 0.092 day^{-1} that applied to all BTCs of PRD1. For MS2, the likelihood ratio testing gave two different values of 0.23 day^{-1} and 0.15 day^{-1}. The higher value applied to the BTCs at PL32, PL23, PL33, PL43 and PD113 (the second well at NAP -7.5 m and all wells at NAP -8.5 m), whereas the lower value applied to the BTCs at PL21, PL31, PL41, PD111, PL22, PL42 and PD112 (all wells at NAP -6.5 m and −7.5 m depth, except PL32).

3.5. Attachment and detachment rate coefficients of MS2 and PRD1

Table 4 presents the estimated parameter values associated with attachment and detachment. For ten of the twelve MS2 BTCs and for eight of the twelve PRD1 BTCs, R^2 values are 79% or above, up to 99%, which is satisfactorily. R^2 values are lower for the BTCs at larger distances and with fewer data that also represent lower virus number concentrations. Note that in general, estimates of k_{a1} and k_{d1} are fairly accurate (standard error one order of magnitude less than the estimate), whereas estimates of k_{a2} and k_{d2} may be quite inaccurate (standard error in the same order of magnitude as the estimate). All BTCs were fitted on the basis of log transformed concentrations, except for the BTC of MS2 at PL31, where no log transformation led to a visually much better fit with a higher R^2.

Based on lowest AIC value as model selection criterion, a model including S_{max2} was not selected for any of the BTCs. For MS2, the one-site kinetic model was selected for wells PL4-1, PD11-1, PL3-2, PL4-2 and PD11-2. The two-site kinetic model was selected for wells PL2-1, PL3-1, PL2-2, PL2-3,PL3-3 and PL4-3. For well PD11-3, only attachment to site 1 needed to be estimated. Estimation of S_{max1} was included for wells PL3-1, PL2-2 and PL3-2.

For PRD1, based on the lowest AIC, the one-site kinetic model was selected for wells PL2-1, PL4-1, PD11-1, PL2-2, PL3-2, PD11-2 and PL5-3. The two-site kinetic model was selected for wells PL3-1, PL4-2, PL2-3 and PL3-3. For wells PL4-1, PL4-3 and PD11-3 only attachment to site 1 needed to be estimated. Estimation of S_{max1} was included for wells PL2-2 and PL3-2.

Table 3
Geochemical analysis of samples of soil and water from the monitoring wells (pH 7.6–7.7).

Well	clay %	silt %	CEC mEq/kg dw	Calcium g/kg dw	Fe-oxalate mg/kg dw	Al-oxalate mg/kg dw	Mn-oxalate mg/kg dw	O_2 mg/l water	Ca^{2+} mg/l water
PL2-1	1.04	1.42	8	34.7	720	82	8.6	1.7	67
PL3-1	0.61	0.74	8	31.1	860	73	6.7	1.5	70
PL4-1	–	–	–	–	–	–	–	1.1	67
PL2-2	1.73	1.35	13	48,5	875	110	12	0.7	70
PL3-2	2.75	3.25	18	45.5	875	115	15	0.7	69
PL4-2	1.21	1.03	10	46.0	870	90	9.6	0.5	68
PL2-3	1.22	1.45	11	41.3	900	94	0.24	0.8	71
PL3-3	3.83	3.4	23	26.9	1120	160	15	0.7	71
PL4-3	1.09	1.35	11	70.1	790	91	15	0.4	60

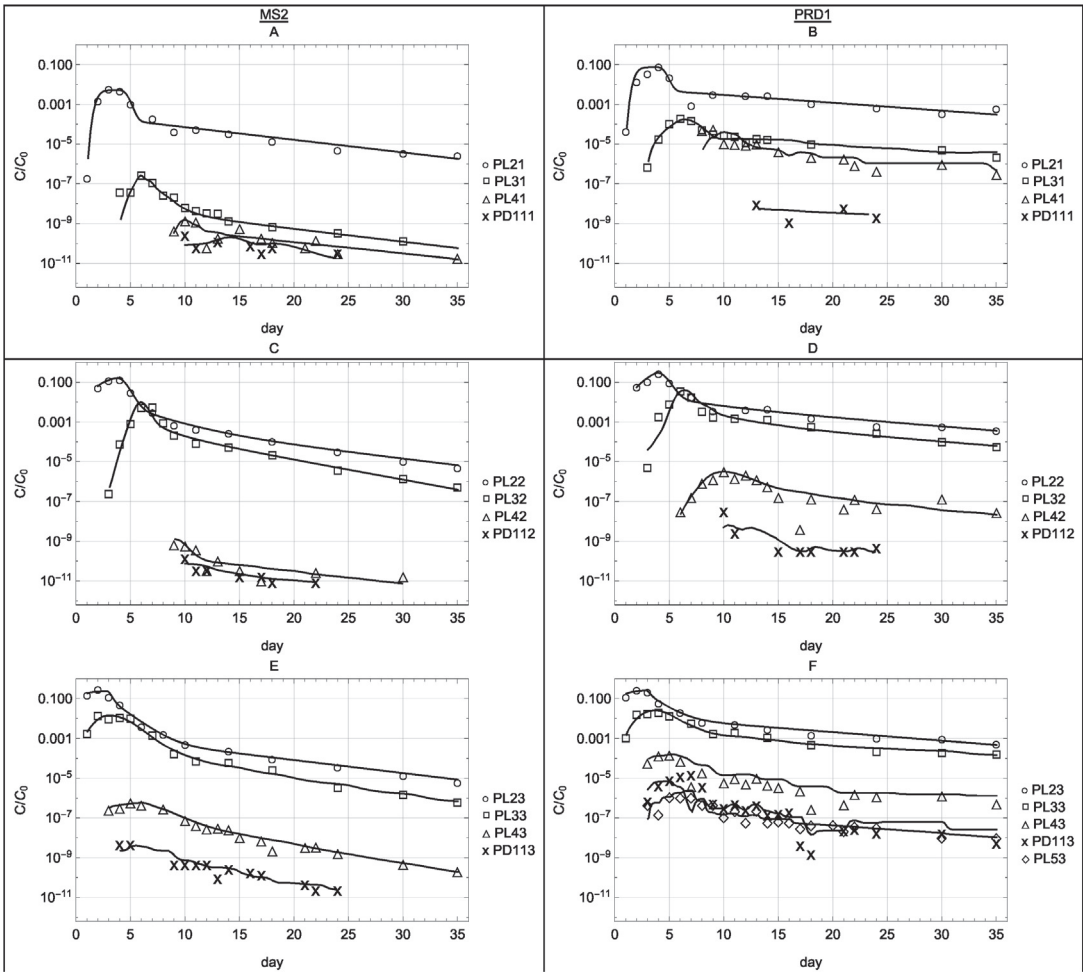

Fig. 4. Breakthrough curves of MS2 (left) and PRD1 (right). A + B: −6.5 m NAP with PL2-1 (2.5 m), PL3-1 (5 m), PL4-1 (10 m), PD11-1 (16 m); C + D: −7.5 m NAP with PL2-2 (2.5 m), PL3-2 (5 m), PL4-2 (10 m), PD11-2 (16 m); −8.5 m NAP with PL2-3 (2.5 m), PL3-3 (5 m), PL4-3 (10 m), PD11-3 (16 m); . Only PRD1 was observed in the monitoring well PL5-3 at 18 m, resulting in five breakthrough curves in this graph.

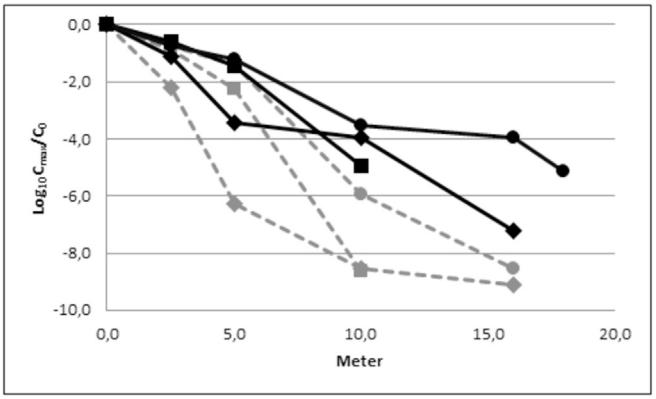

Fig. 5. Virus removal log10 (C_{max}/C_0) of MS2 (grey) and PRD1 (black) versus the travel distance at NAP -6.5 (diamonds), −7.5 (squares) and −8.5 (circles) m below NAP.

The finding that a one-site kinetic model was sufficient to describe a BTC, instead of a two-site kinetic model, may be determined by the shape of the BTC, but also may be due to the fact that the data did not bare enough additional information relative to that from the BTC of the preceding well to make this distinction (fewer data and/or more random error, especially at larger travel distances). Some of the BTCs were described well enough by attachment rate coefficient k_{a1} only. A meaningful estimate of detachment could not be made in these cases. Similarly, the BTCs, for which an estimate of S_{max1} was obtained, indicated that blocking was occurring. Those BTCs, for which an estimate of S_{max1} was not needed, indicated that blocking did not play a role, but absence of blocking is not conclusive. So, blocking was indicated for both bacteriophages at wells PL22 and PL32, with a lower S_{max1} for well PL32 and lower S_{max1} values for PRD1 than for MS2. For MS2, blocking was also indicated for well P31 with an even lower S_{max1} value.

3.6. Relation between geochemistry and sticking efficiency or the attachment rate coefficient

Table 5 lists the variables and their interactions that were are included in the selected regression model for the sticking efficiency and attachment rate coefficient. For both models, the included variables are bacteriophage, clay content, silt content, cation exchange capacity, calcium and the Fe and Mn oxalates. Clay content, silt content, CEC, and content of iron and aluminium oxides are all highly correlated with each other. Best model selection in R accounts for high collinearity. Nevertheless, given the high collinearity, and the limited number of data, predictions of sticking efficiency values was not considered relevant. Here, the regression

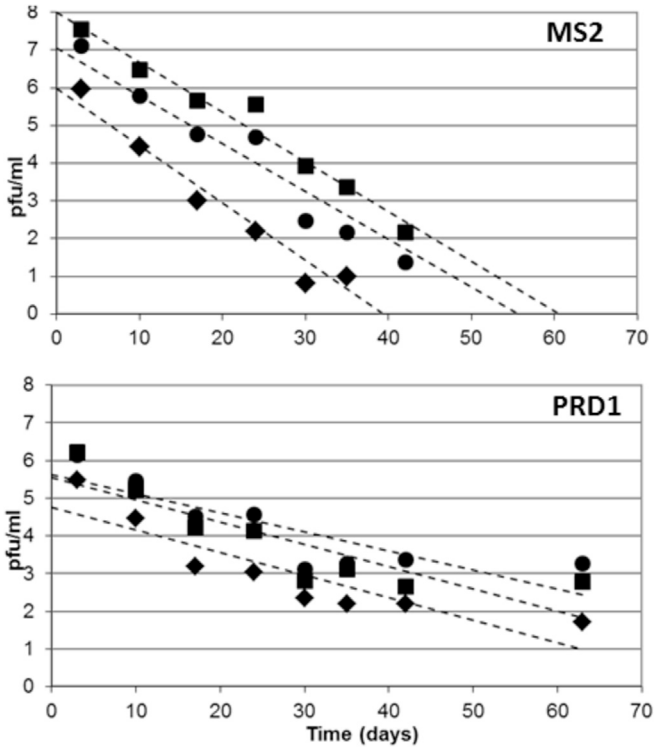

Fig. 6. Inactivation of MS2 and PRD1 at 15 °C in suboxic groundwater collected at NAP -6.5 (diamonds), −7.5 (squares) and −8.5 m (circles).

analysis was only used to distinguish the most important variables affecting attachment expressed in the sticking efficiency. Note that in the model for sticking efficiency, bacteriophage is not a significant term, but is, nevertheless, part of the model selection based on likelihood ratios as selection criterion. In the model for k_{att}, bacteriophages are a significant term, indicating less attachment of PRD1 than of MS2 as apparent in Fig. 5.

Table 5
Results of multivariate regression analysis and best model selection.

Variable	Coefficient		Standard error	*p*
Logit(α) = a_0+ a_1*phagePRD1+a_2*clay + a_3*silt + a_4*cec + a_5*ca + a_6*fe + a_7*mn. R^2 = 85%.				
Intercept	a_0	−28	4.2	***
phagePRD1	a_1	0.27	0.17	
clay	a_2	−0.035	0.0080	**
silt	a_3	0.048	0.0094	***
cec	a_4	−0.68	0.19	**
ca	a_5	140	42	*
fe	a_6	0.024	0.0047	**
mn	a_7	0.24	0.042	***
katt = a_0+ a_1*phagePRD1+a_2*clay + a_3*silt + a_4*cec + a_5*ca + a_6*fe + a_7*mn. R^2 = 80%.				
Variable	Coefficient		Standard error	*p*
Intercept	a_0	−58	16	**
phagePRD1	a_1	−1.7	0.64	*
clay	a_2	−0.12	0.031	**
silt	a_3	0.15	0.037	**
cec	a_4	−1.7	0.75	*
ca	a_5	420	160	*
fe	a_6	0.069	0.018	**
mn	a_7	0.67	0.16	**

phagePRD1 = bacteriophage PRD1, the coefficient applies to PRD1, for MS2 the coefficient is 0; cec = cation exchange capacity; Fe=Fe oxalate; Mn=Mn oxalate. Dimensions as in Table 3; significance level of probability *p*: ≤0.1; *≤0.05; **≤0.01; ***≤0.001.

4. Discussion

The geochemical analyses confirmed that the field study was conducted under suboxic conditions, *i.e.* less than 1 mg/l O_2. At this low oxygen conditions, efficient removal of bacteriophages MS2

Table 4
Attachment and detachment rate coefficient estimates from fitting BTCs.

Well	k_{a1} (day^{-1})		$\alpha \times 10^{-3}$	k_{d1} (day^{-1})		S_{max} ()		k_{a2} (day^{-1})		k_{d2} (day^{-1})		R^2
	EST.	SE.		EST.	SE.	EST.	SE.	EST.	SE.	EST.	SE.	
MS2												
PL2-1	2.3	0.12	0.67	0.0032	0.00069	–	–	13	28	95	243	99%
PL3-1	7.8	0.73	2.3	0.0013	0.00097	289	40	0.22	0.075	1.1	1.0	98%
PL4-1	0.84	0.11	0.25	0.035	0.013	–	–	–	–	–	–	72%
PD11-1	0.021	0.11	0.0062	0.11	1.2	–	–	–	–	–	–	25%
PL2-2	1.1	0.21	0.32	0.0022	0.00026	51 000	12 000	0.037	0.010	0.26	0.11	100%
PL3-2	7.6	0.52	2.2	0.032	0.0073	5300	360	–	–	–	–	97%
PL4-2	4.0	0.034	1.2	0.0032	0.0013	–	–	–	–	–	–	87%
PD11-2	0.79	0.27	0.23	0.068	0.036	–	–	–	–	–	–	79%
PL2-3	1.4	0.24	0.34	0.0019	0.00027	–	–	0.58	0.30	0.98	0.27	99%
PL3-3	2.9	0.30	0.70	0.0027	0.0013	–	–	15	11	6.2	4.9	99%
PL4-3	4.5	0.11	1.1	0.0035	0.00085	–	–	1.1	0.56	1.1	0.66	98%
PD11-3	3.4	0.17	0.83	–	–	–	–	–	–	–	–	88%
PRD1												
PL2-1	1.2	0.21	0.66	0.012	0.0045	–	–	–	–	–	–	92%
PL3-1	2.5	0.078	1.5	0.0054	0.0017	–	–	2.3	0.47	4.5	1.2	97%
PL4-1	0.21	0.060	0.13	–	–	–	–	–	–	–	–	66%
PD11-1	2.2	0.88	1.3	0.015	0.034	–	–	–	–	–	–	9.4%
PL2-2	1.6	0.35	0.93	0.019	0.0035	2500	450	–	–	–	–	98%
PL3-2	4.6	0.71	2.7	0.019	0.0089	690	110	–	–	–	–	84%
PL4-2	2.1	0.075	1.2	0.00057	0.0016	–	–	0.82	0.72	1.7	1.9	75%
PD11-2	3.7	0.61	2.2	0.0051	0.0044	–	–	–	–	–	–	74%
PL2-3	1.4	0.19	0.70	0.016	0.0020	–	–	1.0	0.51	1.2	0.53	99%
PL3-3	2.6	0.25	1.3	0.0075	0.0025	–	–	50	70	21	33	98%
PL4-3	2.1	0.051	1.1	–	–	–	–	–	–	–	–	90%
PD11-3	4.9	0.84	2.4	–	–	–	–	–	–	–	–	72%
PL5-3	5.9	0.95	2.9	0.012	0.0035	–	–	–	–	–	–	83%

EST. = estimated value; SE. = standard error; - = Parameter not part of the model.

and PRD1 was observed in this sandy aquifer. Over a distance of about 16 m 9.1 $\log_{10}$ removal for MS2 and 7.2 $\log_{10}$ removal for PRD1 was demonstrated in the top layer. The lowest layer at NAP -8,5 m showed over this same distance a removal of 8.5 $\log_{10}$ for MS2 and 4.0 $\log_{10}$ for PRD1. The higher pore water velocity in this layer, results in less removal of the bacteriophages, and this is in line with previous studies (Pang, 2009).

Table 4 also lists the estimated sticking efficiency values. For MS2 at well PD11-1, the sticking efficiency is very low: 6.2×10^{-6}. Also, for PRD1 at well PL4-1 it is low: 6.3×10^{-5}. Otherwise, the values of the sticking efficiency range from 0.25×10^{-3} to 2.3×10^{-3} for MS2, and from 0.33×10^{-3} to 1.4×10^{-3} for PRD1. Note that a sticking efficiency of 10^{-3} implies that only one of every thousand collisions between bacteriophage particles and soil grain surfaces results in attachment, but given many collisions, there is enough attachment to cause this efficient removal. With the knowledge that under anoxic conditions, virus removal is poor with associated sticking efficiency values in the order of 10^{-5} and that it is efficient under oxic conditions with sticking efficiency values in the order of 10^{-3} (Schijven et al., 2000), this study adding data about virus removal under suboxic conditions is a meaningful addition to our knowledge. Apparently, under suboxic conditions, virus removal is as efficient as under oxic conditions. This implies that for protection of natural groundwater under suboxic conditions, a setback distance corresponding to a travel time of 60 days is sufficient.

In this study MS2 showed more attachment than PRD1 (Table 4 and Fig. 4). In a previous field study in a dune area close to Castricum it was shown that attachment of MS2 was approximately the same as PRD1 (Schijven et al., 1999, 2000a), and similar sticking efficiencies for both bacteriophages were described. Ionic strength of the water influences attachment of bacteriophages, but not on an identical manner for MS2 and PRD1. At low ionic strength, adhesion of MS2 to a positively charged hydrophobic membrane was higher than PRD1, while at higher ionic strength the opposite was observed (Dika et al., 2015). Comparing the ionic strength of the aquifer water of Castricum (Schijven et al., 2000) with the ionic strength of the aquifer water of this study, shows that the EC in Castricum was 914 $\mu S\,cm^{-1}$, while the EC of the water at the site in this study was 720 $\mu S\,cm^{-1}$. This might favour attachment of MS2 above PRD1, and could explain why attachment of MS2 is higher than PRD1 at this experimental site.

With residence times of about 15 days in the aquifer at NAP -6.5 and −7.5 m, and about 7 days at NAP -8.5 m to the monitoring well at 16 m downstream of injection, only a small part of the total virus reduction is caused by inactivation. A period of 15 days in groundwater of 15 °C, decreases the MS2 population approximately 2 log by inactivation, while the PRD1 population decreases by 0.8 log. Inactivation in water in the aquifer at NAP -6.5 m, containing an oxygen concentration between 1.1 and 1.7 ml/l, proceeded slightly faster than in water with oxygen levels below 1.0 mg/l, at NAP -7.5 and −8.5 (Fig. 6). This is in agreement with previous observations, where inactivation proceeded less fast under anoxic conditions (Frohnert et al., 2014; van der Wielen et al., 2008; Gordon and Toze, 2003).

Predictions of virus attachment with the regression model were not given, because of the limited set of data (twelve sticking efficiency values for each phage) and ten different conditions. The physico-chemical conditions and the parameters describing transport of the bacteriophages (inactivation, attachment, detachment and blocking) indicated heterogeneities in the form of hydraulic conductivity with preferential flow paths at different depths.

Regression analysis identified content of the bacteriophage, clay content and the metal oxides as the most important physico-chemical conditions affecting virus attachment to soil within the range of these conditions of the field study. According to the regression model, bacteriophage PRD1 attaches less than MS2. The calcium content, and iron and manganese oxides are indicated to increase virus attachment (Abudalo et al., 2005). Higher contents of calcium ions and metal oxides are expected to do this by providing favourable sites for attachment (Schijven et al., 2000). It is unclear why this seems to be the opposite for aluminium oxides.

It should be noted that the chemical conditions in this field study did not vary over a wide range and that redox conditions were suboxic or very close to at all monitoring wells. Nevertheless, the physico-chemical conditions and the parameters describing transport of the bacteriophages (inactivation, attachment, detachment and blocking) indicated heterogeneities in the form of hydraulic conductivity with preferential flow paths at different depths. Specifically in the lower layer at NAP -8.5 m, areas containing intact shells were found. Furthermore, around monitoring well PL3, 5 meters downstream of injection, a clearly deviating soil composition was observed, contain a significantly higher clay content at NAP -7.5 and - 8.5 m, but a lower clay content at NAP -6.5 m. Apparently, local heterogeneity can considerably impact the removal of viruses. MS2 is removed more than PRD1, especially between five and ten meters of travel distance (Fig. 5).

5. Conclusions

This study shows that virus removal by soil passage under suboxic conditions is equally effective as under oxic conditions, in contrast to much lower virus removal under anoxic conditions. This implies that for protection of natural groundwater under suboxic conditions, a setback distance similar to oxic conditions and corresponding to a travel time of 60 days is adequate to ensure sufficient protection against virus contamination.

MS2 was removed more efficiently than PRD1. After 16 meter of aquifer transport, 9.1 $\log_{10}$ removal for MS2 and 7.2 $\log_{10}$ removal for PRD1 was demonstrated in the top layer, while over this same distance in the layer located 2 meter below the top layer a removal of 8.5 $\log_{10}$ for MS2 and 4.0 $\log_{10}$ for PRD1 was demonstrated. Apparently, PRD1 is a more conservative model virus than MS2, not only because it inactivates slower, it also attaches less. The lower removal in the lower layer was ascribed to the almost 2 times higher average porewater velocity.

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