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Bacteriophage PRD1 batch experiments to study attachment, detachment and inactivation processes

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ABSTRACT

Knowledge of virus removal in subsurface environments is pivotal for assessing the risk of viral contamination of water resources and developing appropriate protection measures. Columns packed with sand are frequently used to quantify attachment, detachment and inactivation rates of viruses. Since column transport experiments are very laborious, a common alternative is to perform batch experiments where usually one or two measurements are done assuming equilibrium is reached. It is also possible to perform kinetic batch experiments. In that case, however, it is necessary to monitor changes in the concentration with time. This means that kinetic batch experiments will be almost as laborious as column experiments. Moreover, attachment and detachment rate coefficients derived from batch experiments may differ from those determined using column experiments. The aim of this study was to determine the utility of kinetic batch experiments and investigate the effects of different designs of the batch experiments on estimated attachment, detachment and inactivation rate coefficients. The experiments involved various combinations of container size, sand–water ratio, and mixing method (i.e., rolling or tumbling by pivoting the tubes around their horizontal or vertical axes, respectively).

Batch experiments were conducted with clean quartz sand, water at pH 7 and ionic strength of 20 mM, and using the bacteriophage PRD1 as a model virus. Values of attachment, detachment and inactivation rate coefficients were found by fitting an analytical solution of the kinetic model equations to the data. Attachment rate coefficients were found to be systematically higher under tumbling than under rolling conditions because of better mixing and more efficient contact of phages with the surfaces of the sand grains. In both mixing methods, more sand in the container yielded higher attachment rate coefficients. A linear increase in the detachment rate coefficient was observed with increased solid–water ratio using tumbling method. Given the differences in the attachment rate coefficients, and assuming the same sticking efficiencies since chemical conditions of the batch and column experiments were the same, our results show that collision efficiencies of batch experiments are not the same as those of column experiments. Upscaling of the attachment rate from batch to column experiments hence requires proper understanding of the mixing conditions. Because batch experiments, in which the kinetics are monitored, are as laborious as column experiments, there seems to be no major advantage in performing batch instead of column experiments.

1. Introduction

Both groundwater and surface water may become contaminated with pathogenic viruses from various fecal sources (Yates

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et al, 1985). There is worldwide interest in the removal of viruses by soil passage; either for the protection of groundwater supplies, or for treatment of surface water for drinking water. Major processes determining virus removal during soil passage are attachment to and detachment from soil grains, as well as virus inactivation. These processes depend on a range of environmental conditions, as explained in several recent reviews (Bradford et al., 2004; Foppen and Schijven, 2006; Jonczyk et al., 2011; Sen, 2011; Sen and Khilar, 2006).

Three types of experiments are typically conducted to obtain parameter values for virus attachment, detachment and inactivation: batch, column and field experiments. Each type of experiment has its characteristic spatial and temporal scales with certain advantages and disadvantages. A major advantage of field studies is the fact that the actual situation is being investigated, which may involve 1-, 2-, or 3-dimensional transport depending upon local hydrologic conditions. Field studies involving pathogens, or even harmless indicator micro-organisms like bacteriophages, are often not allowed because of safety regulations. Commonly, field studies are laborious and can be very expensive. Physico-chemical heterogeneities further add to the complexity of field-scale studies and may necessitate sampling at multiple locations (see e.g. Pang, 2009; Ryan et al., 1999; Schijven et al., 1999, 2000).

Column experiments offer the advantage that permission from local authorities is not required, and that the transport and removal processes can be studied under well-defined physico-chemical conditions. Column experiments are often used to support field studies. However, because of spatial and temporal variations in field conditions, an extensive series of column experiments may be required in order to encompass field heterogeneities. Also, a large number of samples still may be needed for accurate parameter estimation, thus rendering column experiments laborious (Lewis and Sjöstrom, 2010). An additional challenge is the appropriate upscaling of parameter values from the column to the field scale (Pang et al., 2003). For these reasons, most column studies are aimed at understanding removal processes rather than obtaining parameters applicable to the field scale (e.g. Bradford and Bettahar, 2005; Sen and Khilar, 2006; Stevik, 2004).

Batch experiments can be performed with or without soil. A batch experiment with only water may be used to determine inactivation rates of viruses in the water phase. Rate parameters are then obtained from measurements of the decreasing virus concentration as a function of time. To study attachment and detachment processes, batch experiments are carried out in which a suspension of viruses is agitated with a known quantity of solid material (e.g., a field soil or quartz sand). While agitating the containers, virus particles simultaneously attach onto and detach from the solid grains. Both free and attached virus particles may become inactivated, leading to a gradual decrease in the number of infectious virus particles. Because of inactivation, a true steady-state equilibrium will never be reached, but rather a quasi-equilibrium situation (Grant et al., 1993).

Initially, in a batch experiment, free virus concentrations decline rapidly with time because of attachment and inactivation. Eventually a quasi-equilibrium distribution of viruses between the solid surfaces of the sand and the water is obtained because of reversible adsorption. Because attachment and detachment are kinetic processes, for modeling purposes one needs to obtain values of the attachment and detachment rate coefficients, rather than only the equilibrium distribution coefficient. The distribution coefficient as such is of little use unless augmented with a kinetic rate coefficient.

To determine virus concentrations in the water phase, water and sand need to be separated. This is relatively easy to do in case of coarse sand since the grains will settle quickly as soon as shaking of the container ends, after which a sample from the supernatant water can be taken. In case of medium- and fine-textured materials, centrifugation is required to separate water and soil. Because of these sampling issues, and to maintain saturated conditions, batch experiments usually make use of a series of containers (tubes) that are sampled only once.

Usually only equilibrium batch experiments are carried out. Samples in that case are taken only at the beginning of an experiment and at the end when equilibrium is assumed to be reached. The equilibration time is often assumed to be 30 min to 8 h (Schijven and Hassanizadeh, 2000). Such experiments can be used only to estimate the distribution coefficient. For this reason, only few values for attachment and detachment rate coefficients from batch studies are available. Unfortunately, the results of batch experiments can vary drastically when performed under different experimental conditions, such as the solid–water ratio and the method of agitation (Schijven and Hassanizadeh, 2000). It is therefore not always clear whether results of batch studies can be used to predict virus removal under transport conditions.

In this paper, we investigate whether the gap between batch and column experiments for studying virus attachment, detachment and inactivation can be bridged. The container size, solid–water ratio, and the mixing method were varied in order to determine their effect on attachment, detachment and inactivation. The batch experiments were conducted with the bacteriophage PRD1 as a model virus. In previous work (Sadeghi et al., 2011), attachment, detachment and inactivation were studied in saturated sand columns for a range of pH and ionic strength values. Since we used the same coarse sand and bacteriophage, values of the rate coefficients from the batch and column experiments can be compared immediately.

2. Material and methods

2.1. Experimental set-up and measurements

The bacteriophage PRD1 was used as a model virus in all experiments. PRD1 is an icosahedral phage with a diameter of 62 nm and an isoelectric point between pH 3 and 4, implying that the phage is negatively charged at pH values between 5 and 8 (Loveland et al., 1996). PRD1 may be considered a worst-case model virus because of its low inactivation rate between 10 and 23 °C (Blanc and Nasser, 1996). Because of its larger size, PRD1 is of interest as a representative of rotaviruses and adenoviruses (Sinton et al., 1997). Its host bacterium was *Salmonella typhimurium* LT-2. Host bacteria and the bacteriophage were obtained from the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. For the soil we used quartz sand (H31, Sibelcoo, Belgium) with an average grain size of 0.44 mm. Potential impurities were removed following procedures adopted by Foppen et al. (2007) to clean sand.

Our sand for this purpose was heated to 850 ± 50 °C for 4 h, followed by acid washing with 12 N HCl for 48 h, and subsequent rinsing with de-ionized water until the electrical conductivity of rinse water was less than 1 μS/cm. Prior to use, the sand was oven-dried for 24 h at 105 °C. A stock solution of water with a pH of 7 and ionic strength (IS) of 20 mM was prepared using sodium salts (NaCl, $NaHCO_3$), similarly as for the column experiments of Sadeghi et al. (2011) to enable direct comparisons of the batch and column experiments. A seeding suspension with a PRD1 concentration of 10^8 pfp/ml was prepared. All experiments were conducted at a controlled room temperature of 11 (±1) °C.

For the inactivation experiments we used the stock solution for all batch experiments without soil. The solution was dosed with the same amount of PRD1 from the seeding suspension. Samples were taken regularly over a 2-month period to determine PRD1 concentrations according to ISO 10705-I (ISO, 1995), except for nalidixic acid since our host bacteria *S. typhimurium* LT-2 was sensitive to this acid.

Two series of batch experiments with soil were performed, each with a different mixing method. In each series, four combinations of solid–water ratios were used (see Table 1). Two or ten grams of dried sand was placed into small and large tubes (15 and 50 ml Greiner Bio-One Polypropylene Falcon tubes, Cat No. 188271 and 227261). Subsequently, all tubes were filled with the stock solution and then drained after a few hours. Filling and drainage were repeated several times during a one-week period until constant pH and ionic strength values were obtained, as indicated by measurements after each replacement. The tubes were subsequently inoculated with the same number of viruses (2.2×10^6 viruses). All tubes were gently filled completely so that no air remained in the tubes upon their closure with caps. The tubes were mounted onto two different agitators. For batch series 1 we used an electrical rotor (Watson Marlow 505S) that enabled us to roll the tubes horizontally at 4 rpm (Fig. 1a–1d). For batch series 2, the tubes were tumbled at 9 rpm (Fig. 2a–2d). A total of 104 tubes were prepared for sampling at 13 different times.

At various times, a tube from each batch experiment was taken for sampling. This tube was left standing for one hour to allow the sand to settle. Its supernatant was then sampled to determine the remaining virus concentration. All samples were analyzed within 2 h of collection.

Table 1
Experimental conditions and estimated attachment, detachment and inactivation parameter values.

Experiment	Conditions			Parameter values			
	M_s g	V_w Ml	M_s/V_w g/ml	C_0 pfp/ml	k_{att} h^{-1}	k_{det} h^{-1}	μ_s h^{-1}
1a	2	55	0.036	4.18×10^4	0.19	0.026	0.007
1b	2	15.3	0.13	1.30×10^5	0.086	–	0.007
1c	10	52	0.19	5.02×10^4	0.21	0.014	0.007
1d	10	12.3	0.81	1.72×10^5	1.2	0.0014	0.007
2a	2	55	0.036	3.45×10^4	0.59	0.000027	0.007
2b	2	15.3	0.13	1.41×10^5	1.1	0.00037	0.066
2c	10	52	0.18	3.39×10^4	0.74	0.00091	0.066
2d	10	12.5	0.80	1.37×10^5	2.1	0.0036	0.066

All experiments were performed at pH 7 and ionic strength 20 mM; in series 1, the tubes were rolled at 4 rpm; in series 2, the tubes were tumbled at 9 rpm.

2.2. Conceptual model

In a batch experiment with water, sand and virus particles, the governing equations describing changes in the virus concentrations in the water phase and attached to the sand grains, as a function of time, are:

$$\frac{dC}{dt} = -k_{att}C + k_{\det}\frac{M_s}{V_w}S - \mu_l C \tag{1}$$

$$\frac{dS}{dt} = k_{att}\frac{V_w}{M_s}C - k_{\det}S - \mu_s S \tag{2}$$

where t is time [T], C is the number of free viruses per volume of water [L^{-3}], S is the number of attached viruses per mass of sand grains[M^{-1}], M_s is the dry mass of sand [M], V_w is the volume of water [L^3], k_{att} and k_{det} are the attachment and detachment rate coefficients [T^{-1}] and μ_l and μ_s are the inactivation rate coefficients, of free and attached viruses, respectively [T^{-1}]. Subject to the initial conditions that $C(t = 0) = C_0$ and $S(t = 0) = 0$, the analytical solutions for C and S were derived using the DSolve command of Mathematica (8.0.4) (Wolfram, 2012). They are as follows:

$$C(t) = \frac{\beta_1 \exp(-\beta_4 t) + \beta_2 \exp(-\beta_5 t)}{2\beta_3} \tag{3}$$

$$S(t) = \frac{\beta_6 \exp(-\beta_4 t) + \beta_7 \exp(-\beta_5 t)}{2\beta_3} \tag{4}$$

where

$$\beta_1 = C_0(k_{att} + \mu_l - k_{\det} - \mu_s + \beta_3) - 2k_{\det}\frac{M_s}{V_w}S_0 \tag{5}$$

$$\beta_2 = -C_0(k_{att} + \mu_l - k_{\det} - \mu_s - \beta_3) + 2k_{\det}\frac{M_s}{V_w}S_0 \tag{6}$$

$$\beta_3 = \sqrt{{\beta_8}^2 - 4\beta_9} \tag{7}$$

$$\beta_4 = \frac{\beta_8 + \beta_3}{2} \tag{8}$$

$$\beta_5 = \frac{\beta_8 - \beta_3}{2} \tag{9}$$

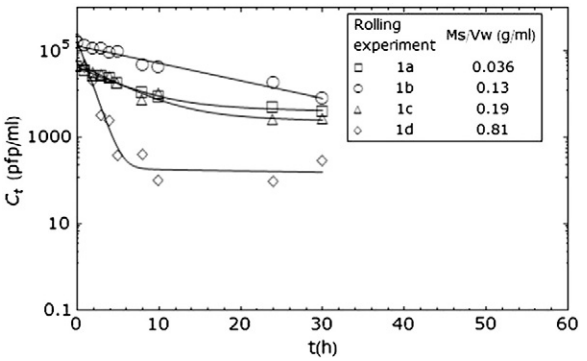

Fig. 1. Evolution of PRD1 concentration in experimental series 1 (rolling at a frequency of 4 rpm). Symbols are observed virus concentrations and lines are fitted model results.

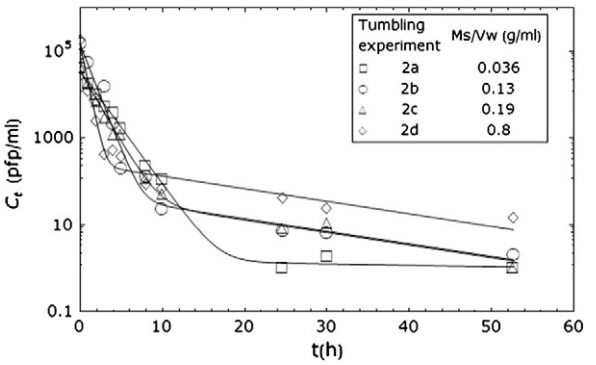

Fig. 2. Evolution of PRD1 concentration in experimental series 2 (tumbling at a frequency of 9 rpm). Symbols are observed virus concentrations and lines are fitted model results.

$$\beta_6 = S_0(k_{\text{det}} + \mu_s - k_{att} - \mu_l + \beta_3) - 2k_{att}\frac{V_w}{M_s}C_0 \tag{10}$$

$$\beta_7 = -S_0(k_{\text{det}} + \mu_s - k_{att} - \mu_l - \beta_3) + 2k_{att}\frac{V_w}{M_s}C_0 \tag{11}$$

$$\beta_8 = k_{att} + k_{\text{det}} + \mu_l + \mu_S \tag{12}$$

$$\beta_9 = k_{att}\mu_S + k_{\text{det}}\mu_l + \mu_l\mu_S. \tag{13}$$

Eqs. (3) and (4) show that the concentrations of both free and adsorbed viruses eventually approach zero because of inactivation.

2.3. Data analysis

The main parameters in Eqs. (3)–(4) are C_0, M_s, V_w, k_{att}, k_{det}, μ_l, and μ_s. Values of C_0, M_s, and V_w were measured directly. In all cases, the value of μ_l was set equal to 0.007 h^{-1}. This value was determined from batch experiments without sand, assuming first-order kinetic inactivation.

Coefficients k_{att}, k_{det} and μ_s were determined by fitting Eq. (3) to observed concentration data. To that aim, the following log-likelihood function was used (Hogg and Craig, 1995):

$$L[k_{att}, k_{\text{det}}, \mu_s] = -2\sum_{i=1}^{n}\left[\ln\left(s\sqrt{2\pi}\right) + \frac{(\log_{10}C_t)^2}{2s^2}\right] \tag{14}$$

where i is the i-th observation out of n observations, C_t is the concentration as a function of time as given by Eq. (3) and s is the normal distributed random error. Parameter values were obtained by minimizing the log-likelihood function using the numerical optimization option of Mathematica (8.0.4) (Wolfram, 2012).

Note that the log transform of the virus concentration was used in the optimization. This was essential since the concentration values covered several orders of magnitude. Without the log transformation, the optimization would be biased almost exclusively towards values at the beginning of the experiments when virus concentrations are still high. For comparison, kinetic parameters were also optimized using concentrations that were not log transformed. For some experiments we obtained very similar values for k_{att} and k_{det} irrespective of the transformation. In several experiments, however, the optimized values differed considerably. This was especially the case for experiments that reached quasi-equilibrium. When the concentrations were not log transformed, the optimized model then did not capture the transition between initial and quasi-equilibrium phase very well. For this reason, only the optimized parameters for the log transformed concentrations are reported here.

In the initial phase of a batch experiment, the decrease in aqueous phase concentrations is mainly controlled by k_{att} and μ_l. But later, when quasi-equilibrium is reached, the decrease in concentration of free viruses is determined by inactivation of both attached and free viruses. For experiments where the virus concentration decreased below the detection limit before quasi-equilibrium was reached, it was not possible to estimate a value for μ_s. For those experiments, μ_s was set equal to the liquid phase inactivation coefficient of 0.007 h^{-1}, in which case only k_{att} and k_{det} needed to be estimated.

First, we carried out an optimization in which the objective function (Eq. 14) consisted of the sum of all eight log-likelihood functions (one for each experiment). In this way, values of the rate coefficients (k_{att}, k_{det}, and μ_s) could be estimated separately for each experiment. Next, similar log-likelihood functions were defined whereby parameter values of different experiments (or groups of experiments) were assumed to be the same. Using the log-likelihood deviates (the same as the likelihood ratios), we could then determine whether those assumptions were correct. If the log-likelihood between models differed less than the 95-percentile of a χ^2-value, with the degree of freedom equal to the difference in the number of parameters between models, then the model with the least number of parameters was used. We further used multivariate regression analysis to investigate in what manner the batch rate parameters were related to the parameters M_s and V_w.

3. Results

Figs. 1 and 2 show observed and calculated virus concentrations versus time for all eight batch experiments. As explained in the previous section, the model was fitted to the log-transformed bacteriophage concentrations. The fitted parameter values are presented in Table 1.

In all experiments, concentrations of free viruses dropped rapidly at the beginning (Figs. 1 and 2). Depending upon the M_s/V_w ratio, concentrations changed by up to four orders of magnitude within the first 10 h of the experiments. This time period reflects the initial phase in which changes in the virus concentrations are predominately caused by an imbalance between the rates of virus attachment and detachment. During the second phase, rates of virus attachment and detachment are almost balanced and the system reaches quasi-equilibrium with respect to virus attachment. In this quasi-equilibrium phase, the decrease in free virus concentration is predominately due to inactivation. Quasi-equilibrium was not reached in experiment 1b (Fig. 1).

When estimating the rate coefficients for each of the eight batch experiments separately, it became apparent that values of μ_s could be estimated for experiments 1d, 2a, 2b, 2c and 2d only, where quasi-equilibrium was established. In the case of experiments 1a and 1c, quasi-equilibrium was just reached,

and μ_s was estimated but with low certainty. For experiment 1b, quasi-equilibrium was not reached and, hence, only k_{att} could be estimated. In the likelihood ratio test, the estimated values of μ_s for experiments 1b, 1c, 1d and 2a were found not to be significantly different from the default value of 0.007 h^{-1} for free viruses. For experiments 2b, 2c and 2d, however a much higher value of 0.066 h^{-1} (almost the same for all three experiments) was estimated. Thus, depending upon the experimental conditions, there are two very distinctive estimates of μ_s. The higher value was obtained for M_s/V_w ratios equal to or greater than 0.13 g/ml, and for tumbling at 9 rpm.

4. Discussion and conclusions

Batch experiments can be used to obtain information on virus attachment to sand grain surfaces. Results of this study show that the method of mixing has a very substantial effect on virus attachment. We believe that complete mixing of the sand and water was not achieved in the rolling experiments since much of the sand remained in the lower part of the tube, while water was occupying the remainder of the tube. In this case, viruses had to move (by diffusion and some advection) from the water-only region to the water–sand region before they could attach. Much better mixing was achieved by tumbling the tubes. More complete mixing implies fewer mass transfer limitations and hence more immediate physical contact between the free viruses in solution and the sand grains. For experiment 1d, under rolling conditions, virus attachment was of a similar order of magnitude as for the tumbling experiments. This may be ascribed to the high sand–water ratio, which provides more surfaces for attachment and a better distribution of the sand within the tube, and hence better mixing leading to more complete attachment.

Fig. 3 presents k_{att} values as a function of M_s/V_w, where a linear relation between M_s/V_w and k_{att} is apparent. A multivariate regression analysis showed that the increase in k_{att} due to an increase in M_s/V_w was the same for both series of experiments. In the case of experiment series 1, the intercept was not significantly different from zero (Table 2). This is important because if there is no sand in the container, there cannot be any attachment to sand. The linear trend for experiment series 2, however does not go through the origin. There is a steep rise from 0 to about 0.6 h^{-1}in the M_s/V_w value range of 0–0.036 g/ml. So, the relationship is nonlinear. In both series of experiments, more sand in the container leads to an increase in k_{att}.

Fig. 4 presents k_{det} values as a function of M_s/V_w. The data here also seem to indicate a linear relationship, but with different trends for the two series of experiments. Multivariate regression analysis revealed a significant correlation with M_s/V_w, whereas in series 2, the opposite trend was found. Series 1 exhibited a decrease in k_{det} with increasing M_s/V_w, whereas the opposite occurred for series 2. In the latter case, the intercept was not significantly different from zero. Again, obviously, there cannot be detachment from the sand if there is no sand in the container. For series 1, the high k_{det} values at low solid–water ratio are questionable. The estimates for those experiments (1a and 1c) were inaccurate because of a very gradual transition from the kinetic phase to quasi-equilibrium, and the limited number of observations in the quasi-equilibrium stage.

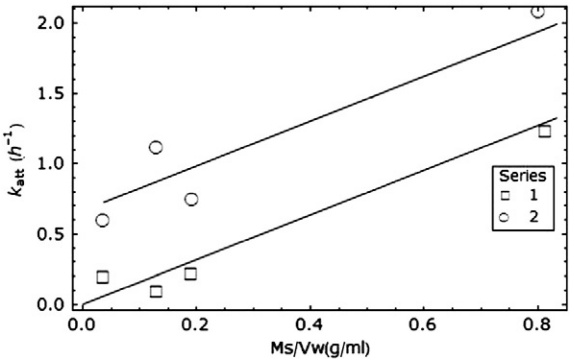

Fig. 3. Attachment rate coefficient k_{att} as a function of M_s/V_w.

Notice that the k_{det} value of experiments 1d and 2d for rolling and tumbling, respectively, was similar for the highest solid–water ratio case. This again can be attributed to the distribution of sand in the water phase. The linear increase in the value of k_{det} with increasing solid–water ratio during tumbling may be ascribed to an increase in hydrodynamic forces or higher collision frequencies between sand particles. This would facilitate virus detachment at the higher solid–water ratio. Also, with the tumbling method of mixing, a larger value of μ_s was obtained. This was specially so for higher M_s/V_w ratios. This suggests that hydrodynamic forces may be damaging virus particles for those conditions.

Another important finding relates to the time required for reaching quasi-equilibrium. Fig. 2 demonstrates that this time is shorter for larger solid–water ratios. For experiment 2a, the equilibrium time was about 20 h, but for experiment 2d only about 4 h. This also means that for batch experiments with viruses, soil and water, it is important to collect samples during both kinetic and quasi-equilibrium phases of an experiment. Moreover, to accurately determine k_{det} as well as μ_s, a sufficient number of samples during the quasi-equilibrium phase is required.

The variability in kinetic parameters as a function of M_s/V_w ratio and agitation method indicates that obtaining kinetic parameters of virus removal in porous media from batch experiments is not straightforward. Our study suggests that performing kinetic batch experiments is as laborious as column experiments with regard to set up, operation, sampling and parameter estimation.

Another important question is how to upscale results of batch experiment to column and larger scales. In this regards, we compared our batch results with parameter values obtained from column experiments at the same conditions, i.e., pH 7 and ionic strength of 20 mM (Sadeghi et al., 2011).

Table 2
Regression analysis of k_{att}, k_{det} as a function of M_s/V_w.

	k_{att}		k_{det}	
	Series 1	Series 2	Series 1	Series 2
Intercept (h^{-1})	0	0.67	0.024	0
Slope (h^{-1} g^{-1} ml)	1.59	1.59	−0.029	0.0046
R^2	94.3%		94.4%	

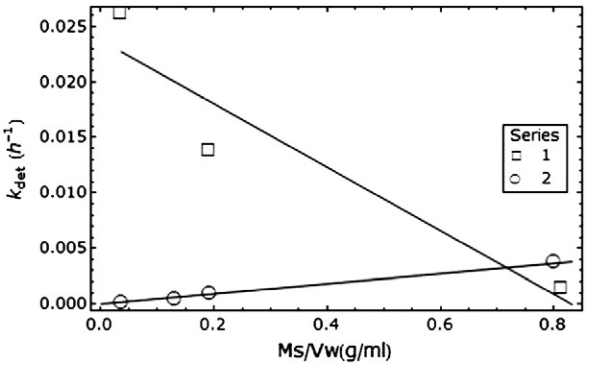

Fig. 4. Detachment rate coefficient k_{det} as a function of M_s/V_w.

The value of k_{att} from those experiments was 0.21 h^{-1}, which is lower than all k_{att} values obtained from the batch experiments under tumbling conditions. Given the fact that the chemical conditions were the same, the sticking efficiency of the column and batch experiments may be assumed to be the same too. This implies that hydraulic conditions in column and batch experiments differ. Thus, the probability of collision of virus particles with the sand grains in batch experiments is much higher than in a sand column. This may seem contradictory to the fact that M_s/V_w ratios in column experiments are almost always higher than in batch experiments. However, the solid phase in column experiments is stationary and hence the frequency of collision of viruses with soil grain surface is lower. This should be the main reason why k_{att} values are lower in column experiments. Another important shortcoming of kinetic batch experiments is that they do not provide any information about the possible dependence of kinetic rate coefficients on the flow velocity, since there is no flow. Column experiments, however, may be designed to provide such information.

The usual practice of determining distribution coefficients from batch experiments is questionable for viruses. Such experiments are commonly based on one single measurement of the virus concentration after only a few hours, thus assuming that equilibrium is reached and that virus inactivation is negligible. Our results show that much longer times are needed for quasi-equilibrium to be reached. Inactivation in that case cannot be neglected anymore. Hence, the proper way of performing batch experiments with viruses requires determining virus concentrations for an extended period of time, and evaluating all attachment and inactivation rate coefficients. This means that well-executed batch experiments are as laborious as column experiments. As such, we conclude from our study that there are no major advantages of doing batch experiments for viruses as compared to column experiments.

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