

Long-term inactivation of bacteriophage PRD1 as a function of temperature, pH, sodium and calcium concentration



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ABSTRACT

The two most significant processes controlling virus mobility in the subsurface environment are virus attachment and inactivation. In particular, models that predict subsurface virus transport are highly sensitive to inactivation. Virus inactivation is known to depend on temperature as well as hydrochemical conditions. The aim of the current work was to study the effects of temperature and hydrochemical conditions on the inactivation of bacteriophage PRD1 as a model virus, and to develop a quantitative relation for these effects. Series of batch experiments under controlled temperature were conducted, for a range of conditions: 9.5 °C and 12 °C, pH4 – pH8, sodium concentrations of 1, 10 and 20 mM, and calcium concentrations of 0.5, 1.5, and 3 mM. By multivariate regression analysis, a joint log-square model was developed that describes the inactivation rate of PRD1 as a function of these hydrochemical conditions. This model approximates two rate and Weibull models and accounts for the observed non-linear inactivation at increased pH and salt concentrations. Model predictions are within $\pm 0.4 \log_{10}$ (0.4–2.5 times) virus concentration reduction. The nature of the log-square model does not allow extrapolation of virus inactivation beyond the experimental conditions. Inactivation rate of PRD1 was found to increase with increasing temperature and increasing sodium and calcium concentrations, and to be lowest between pH 6.5 and pH 7.5. Within the studied conditions, the developed log-square model may be applied at field scale for predicting inactivation during subsurface transport of viruses.

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1. Introduction

Contamination of groundwater with human pathogenic viruses is a significant public health problem in developed and developing countries (Howard et al., 2006). Virus mobility in the subsurface environment is determined by advection, dispersion, dilution, attachment and inactivation. The size of a groundwater protection zone or the removal efficiency of riverbank filtration or dune infiltration highly depends on virus attachment and inactivation rates (Schijven et al., 2006). From a precautionary principle, predictions on virus transport should be based on conservative values for attachment and inactivation. These values were 10^{-5} for the sticking efficiency (virus attachment) and about 0.023 d^{-1} for the average first-order inactivation rate coefficient (Schijven et al., 2006).

Both attachment and inactivation of viruses are dependent on the physico-chemical conditions as well as on the type of virus. Less conservative values for attachment and inactivation may be used if their relation with the physico-chemical conditions is known. Reliable prediction of virus removal by attachment and inactivation requires quantitative relations between physico-chemical conditions and these removal processes. Because of the obstacles in studying pathogenic viruses, such as the hazard level, enumeration limitations, and the large variability in properties determining attachment to solid surfaces and inactivation, bacteriophages are used as model viruses. Bacteriophages are harmless and easy to enumerate (Schijven and Hassanizadeh, 2000). Because of the large variability in virus characteristics, bacteriophages are used that either attach poorly or that inactivate slowly, or that have both these characteristics. Bacteriophage PRD1 has shown to fulfill these requirements in many studies (Harvey and Ryan, 2004; Schijven and Hassanizadeh, 2000).

The effects of pH, ionic strength (IS), and calcium concentration on the attachment of bacteriophages have been studied through

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different batch and column experiments (e.g. Fontes et al., 1991; McCarthy et al., 2002, McCarthy and McKay, 2004; Sadeghi et al., 2011, 2013a, b; Torkzaban et al., 2006). Sadeghi et al. (2011) showed that the attachment rate coefficient and sticking efficiency increase with decreasing pH and increasing IS, which is in agreement with the DLVO theory and the vast literature on these effects. They developed an empirical formula for sticking efficiency as a function of pH and IS. Using this formula within the calibrated range of pH and IS, predicted and observed sticking efficiencies from field and column experiments were found to be in reasonable agreement for bacteriophages PRD1 and MS2. Sadeghi et al. (2013a) also developed an empirical formula for sticking efficiency as a function of calcium concentration.

Virus inactivation occurs because of the disruption of viral coat proteins and the degradation of virus nucleic acids (Harvey and Ryan, 2004; Gerba, 1984). Virus inactivation may occur with viruses suspended in water as well as viruses adsorbed onto surfaces (Sobsey et al., 1980). Inactivation is usually modelled as a first-order process and characterized by an inactivation rate coefficient, but it was apparent from long-term observations that inactivation may not proceed as first order rate process, but may slow down in time and, for example, a biphasic inactivation function is needed to describe this time dependency (De Roda Husman et al., 2009). Knowing such long-term behaviour is crucial in risk assessment, first order rate models based on short-term inactivation data may hugely underestimate virus concentrations after longer times of subsurface transport.

Important factors that influence virus inactivation rates during subsurface transport are temperature (Hurst et al., 1980; Yates et al., 1985, 1987; Jansons et al., 1989; Nasser et al., 1993; Blanc and Nasser, 1996; Harvey and Ryan, 2004), adsorption to particulate matter (Gerba, 1984), soil microbial activity (Hurst, 1988; Nasser et al., 2002; Davies et al., 2006), presence of metal oxides (Chu et al., 2000; Schijven and Hassanizadeh, 2000), calcium concentration (Yates et al., 1985), pH (Feng et al., 2003; Yates et al., 1987) and soil moisture (Song et al., 2005; Yates et al., 1987). Recently, Bertrand et al. (2012) published a meta-analysis of virus inactivation and virus genome degradation data from literature. A linear model was employed by Bertrand et al. (2012) to analyse the effects of temperature, virus species, detection method (cell culture or molecular methods), simple matrix (drinking water, groundwater, synthetic buffer) or complex matrix (seawater, freshwater, sewage, food, soil, biologic fluid and dairy products) and temperature range (<50 °C and ≥50 °C). Obviously, virus inactivation is much higher at higher temperatures (≥50 °C), but there is also a significant temperature-matrix effect. Virus inactivation appeared to occur faster in complex matrices. Virus genome was shown to be more persistent than virus infectivity. From the study of Bertrand et al. (2012), bacteriophages PRD1 and φX174 appeared to be highly persistent under most conditions, which implies they are precautionary indicators for virus inactivation studies. A number of studies exist on the temperature dependence of the inactivation of PRD1 (Anders and Chrysikopoulos, 2006; Charles et al., 2008; Davies et al., 2006; Straub et al., 1992; Yahya et al., 1993), encompassing 4–40 °C, but there are no systematic studies on the effects of solution chemistry on the inactivation of PRD1. In the Netherlands, groundwater tables are shallow and groundwater temperature varies between 9.5 °C and 12 °C (Schijven et al., 2006), representing the temperate climate that can be found in many parts of the world. Higher temperatures are not relevant for the Dutch situation. The setback distances that Schijven et al. (2006) calculated to sufficiently protect shallow sandy aquifers against virus contamination were about 200–400 m, corresponding to travel times of 1–2 years. Therefore, long-term inactivation of viruses needs to be considered. As De Roda Husman et al. (2009) have demonstrated, on the long

term, virus inactivation may not proceed at first order rate but may slow down as time passes.

The aim of the current study is to gain quantitative insight in the effects of hydrochemical and long-term conditions on the inactivation of PRD1 in water under temperate climatic conditions. To that aim, batch experiments were conducted in order to determine the inactivation rate of PRD1 in water for a wide range of ionic strength (IS), pH, and calcium concentrations representative of natural groundwater conditions. Results were used to develop quantitative relationships in order to be able for the estimation of PRD1 inactivation under various hydrochemical conditions in groundwater as part of fate and transport models. This is also essential for predicting setback distances of groundwater protection zones.

2. Material and methods

2.1. Inactivation experiments

In total, twenty inactivation experiments (A – T) were conducted partially in parallel with column and batch experiments. Some of these data were published in Sadeghi et al. (2011, 2013a, b), as follows: Experiments A – E were used in Sadeghi et al. (2011). In Sadeghi et al. (2013a) the data of experiments R – T were used. The data in Sadeghi et al. (2013b) encompass only the first five data points of experiments N. In all these publications (Sadeghi et al., 2011, 2013a, b), those data were used only to estimate the inactivation rate coefficient according to first order rate inactivation, in order to fit the breakthrough curves from the column experiments and the sorption data from the batch experiments. Thus, the new data of experiments F – M, O – Q, and six additional data points in experiment N have never been published or used in publications. These data span three weeks up to nine months. Solutions of NaHCO₃, NaCl and CaCl₂ with a pre-specified pH and IS were prepared for the experiments. The amount of NaHCO₃ for achieving a given pH in equilibrium with atmospheric CO₂ pressure was calculated using MINEQL + 4.6 (<http://www.mineql.com/>). NaCl was added to adjust IS. Prior to inoculation with bacteriophage, the solutions were equilibrated open to the atmosphere over several days and pH was regularly readjusted with NaOH or HCl. The added amounts of NaOH and HCl did not alter IS significantly.

Prevailing conditions for each experiment are given in Table 1. In experiments A – E, pH ranged from pH 4 – pH 8 at a constant sodium concentration of 1 mM. In experiments F – Q, the combined effects of pH (pH 5 – pH 8) and sodium concentration (1, 10 and 20 mM) on inactivation were investigated. In experiments R – T, the effect of calcium concentration (1, 1.5 and 3 mM) at the same IS was studied. Experiments A – E were conducted at 12 ± 0.5 °C and experiments F – T at 9.5 ± 0.5 °C. Note that these ranges of conditions (temperature, pH, sodium and calcium concentration) are representative for Dutch groundwater shallow aquifers (Sadeghi et al., 2011).

A suspension of 10¹³ plaque forming particles per ml (pfp/ml) of PRD1 was prepared as described in ISO 10705-1 and stored at 5 °C ± 3 °C. *Salmonella typhimurium* LT-2 was the host bacteria for PRD1. Host bacteria and bacteriophage were obtained from the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. For each inactivation experiment, seeding suspensions of PRD1 were prepared from the stock suspension by diluting with a solution of pre-specified pH and IS, as described above.

Inactivation of PRD1 in water, defined as the gradual loss of the ability to infect its bacterial host, was measured in batch experiments at two different temperatures (9.5 °C and 12 °C). Initial concentrations (C₀) were about 4 × 10³ pfp/ml in experiments A –

Table 1
Experimental conditions.

Experiment	pH	NaHCO ₃ mM	NaCl mM	CaCl ₂ mM	IS mM	°C	N	Duration days	Best model
A	4	0	1.00	0	1	12	6	19	1
B	5	0	1.00	0	1	12	6	19	1
C	6	0.01	0.99	0	1	12	6	19	1
D	7	0.07	0.93	0	1	12	6	19	1
E	8	0.72	0.28	0	1	12	6	19	1
F	5	0	1.00	0	1	9.5	12	274	1
G	5	0	10.00	0	10	9.5	12	274	1
H	5	0	20.00	0	20	9.5	12	274	1
I	6	0.01	0.99	0	1	9.5	12	274	1
J	6	0.01	9.99	0	10	9.5	12	274	1
K	6	0.01	19.99	0	20	9.5	12	274	3
L	7	0.07	0.93	0	1	9.5	12	274	4
M	7	0.08	9.92	0	10	9.5	12	274	4
N	7	0.08	19.92	0	20	9.5	12	274	2
O	8	0.72	0.28	0	1	9.5	13	274	4
P	8	0.76	9.24	0	10	9.5	13	274	2
Q	8	0.79	19.21	0	20	9.5	13	274	4
R	7	0.08	8.42	0.5	10	9.5	12	184	2
S	7	0.08	5.42	1.5	10	9.5	7	100	2
T	7	0.08	0.92	3	10	9.5	11	170	4
Total number (N) of measurements								208	

Best model 1: one-rate; 2: two-rate; 3: Weibull; 4: log-square.

E, about 10^5 pfp/ml in experiments F – Q, and about 5×10^5 pfp/ml in experiments R – T. The samples were assayed using the plaque forming technique described by ISO 10705-1 (1995), with the omission of nalidixic acid. All samples were analyzed in duplicate within 2 h of collection. In all series of bacteriophage enumerations, blank control and positive quantitative controls were included. The counts of all positive controls were used to construct Shewart control charts to warrant constant quality of media and incubation conditions. Table 1 lists the duration of the experiments.

2.2. Analysis of data

Virus inactivation is commonly described by a first-order inactivation model (Schijven and Hassanizadeh, 2000):

$$\ln C_t = \ln C_0 - \mu_1 t + \varepsilon \quad (1)$$

where C_t is the virus concentration [number of viral particles per ml] at time t [day], C_0 is the initial virus concentration, μ_1 is the virus inactivation rate coefficient [day^{-1}], and ε is random error that follows a Normal distribution with mean equal to zero and standard deviation s . This first order rate model applies if the logarithm of the virus concentration declines linearly in time. The decline in log virus concentration that slows down in time can be described by a biphasic or two rate model, a Weibull model or a log-square model. The two rate model assumes that two sub-populations of the virus particles inactivate independently at different rates (Cerf, 1977):

$$\ln C_t = \ln C_0 + \left[f e^{-\mu_1 t} + (1-f) e^{-\mu_2 t} \right] + \varepsilon \quad (2)$$

where f is the fraction of the virus population for which the virus inactivation rate coefficient μ_1 applies and $(1-f)$ is the fraction for which the virus inactivation rate coefficient μ_2 applies.

The Weibull model assumes that a continuous distribution of inactivation rate coefficients applies (Van Boekel, 2002):

$$\ln C_t = \ln C_0 - (\mu_1 t)^\beta + \varepsilon \quad (3)$$

where β is the shape parameter of the Weibull distribution.

The log-square model also captures the curvature in the decline of logarithmic concentrations by the addition of a second degree term (Stone et al., 2009):

$$\ln C_t = \ln C_0 - \mu_1 t - \mu_2 t^2 + \varepsilon \quad (4)$$

The governing equation for an advection-dispersion model, including exchange between the water phase and the surfaces of the soil grains (attachment and detachment), and inactivation of free and attached viruses is as follows (eg. Schijven and Hassanizadeh, 2000):

$$\frac{\partial C}{\partial t} + \frac{\rho_B}{n} \frac{\partial S}{\partial t} = \alpha_L v \frac{\partial^2 C}{\partial x^2} - v \frac{\partial C}{\partial x} - r_{inac}^f - r_{inac}^s \quad (5)$$

where C [# of virus particles L^{-3}] is the number density of viruses in water, S [# of virus particles M^{-1}] is the number of attached viruses per unit mass of soil, ρ_B [ML^{-3}] is the dry bulk density, α_L [L] is dispersivity, v [LT^{-1}] is the pore water velocity, n [-] is the porosity, and r_{inac}^f and r_{inac}^s are the inactivation rate terms for free and attached bacteriophages, respectively. The formulas for the inactivation rate term for free bacteriophages, corresponding to the different inactivation models (Equations (1)–(4)) in Equation (5) are given below.

For Equation (1):

$$r_{inac}^f = -\mu_1 C \quad (6)$$

For Equation (2):

$$r_{inac}^f = -\left(\mu_1 f e^{-\mu_1 t} + \mu_2 (1-f) e^{-\mu_2 t} \right) C \quad (7)$$

For Equation (3):

$$r_{inac}^I = -\beta\mu_1^\beta t^{\beta-1}C \tag{8}$$

For Equation (4):

$$r_{inac}^I = -(\mu_1 + 2\mu_1 t)C \tag{9}$$

Obviously, the first-order inactivation model is a special case of each of the other three models. In order to investigate whether inactivation proceeded first-order or in a non-linear fashion, likelihood ratio tests were conducted for each of the twenty inactivation experiments using the following log likelihood function (Hogg and Craig, 1995):

$$L[params, s] = 2 \sum_{i=1}^N \left[\ln(s\sqrt{2}) + \frac{(\ln C_i - f(params, t_i))^2}{2s^2} \right] \tag{10}$$

where *params* designates the parameters of inactivation function *f*, representing the first order rate model, the two rate model, the Weibull model or the log-square model. If the difference between the log-likelihood of the first-order rate model and one of the other models is less than the 95-percentile of the Chi-square distribution with one or two degrees of freedom, depending on comparing a one-parameter model with a two- or three-parameter model, respectively, then the first order rate model described inactivation sufficiently. If the first order rate model did not apply, the two rate model, Weibull model and log-square model were compared by their AIC (Akaike Information Criterion) values (Anderson, 2004) and the model with the lowest AIC was considered the best model.

The log-square model was used to develop an inactivation model that describes all the inactivation data jointly as a function of the hydrochemical conditions. This implies that parameters μ_1 and μ_2 were expressed as functions of temperature, pH, sodium and calcium concentration. The development of this joint model was conducted using the linear model function *lm* of the statistical package R (version 3.2.2):

$$lm(\ln(C) \sim \text{expnr} + (T^*pH*\ln(Na)*Ca):t + (T^*pH*\ln(Na)*Ca):I(t^2) + I(T^2):t + I(pH^2):t + I(\ln(Na)^2):t + I(Ca^2):t, \text{data} = \text{obs}) \tag{11}$$

where *expnr* (*A – T*) was included in order to enable estimation of the intercept $\ln C_0$ of each experiment. When using the measured $\ln C_0$ values as fixed values would introduce unwanted systematic error in the data. Obviously, *T*, *pH*, *Na* and *Ca* are the variables that represent the values of temperature, pH, sodium and calcium concentration. Interactions of two variables (or effects) in R are designated by a “:”. For example, *T:t* is the interaction between temperature and time. *T** represents the main effects of temperature and time, including their interaction, *T + t + T:t*. The terms with the conditions squared, eg. *I(T^2):t*, were included to account for possible nonlinear effects of the conditions on the value of μ_1 . The *step* function of R was used to perform model selection, in which terms were removed based on AIC comparison, with parameter *k* = 3.84. 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.

3. Results

Fig. 1 presents the inactivation data of the twenty experiments. According to the likelihood ratio tests, the first order rate model adequately described the data of experiments A – J (Table 1). However, for the other experiments, inactivation was non-linear.

According to model selection on the basis of AIC, the two rate model was the best model to describe the data of experiments N, P, R and S, so was the Weibull model the best model for experiment K and the log-square model the best model for experiments L, M, O, Q and T. The decline of the log concentrations with time was progressively stronger non-linear when pH increased in combination with, especially, increasing sodium concentration.

Fitting all data in order to derive a joint log-square model (Equation (4)), the following formulas for parameters μ_1 and μ_2 as functions of the hydrochemical conditions were obtained:

$$-\mu_1 = a_1T + a_2pH + a_3 \ln Na + a_4Ca + a_5TpH + a_6pH \ln Na + a_7T^2 + a_8pH^2 \tag{12}$$

$$-\mu_2 = b_1pH + b_2 \ln Na + b_3pH \ln Na \tag{13}$$

Model predictions with the joint log-square model, including 95%-prediction intervals (prediction uncertainty), are shown for each experiment in Fig. 1.

Table 2 provides the estimated values of coefficients $a_1 - a_8$ and $b_1 - b_3$. Taking the logarithm of the sodium concentration slightly improved the fitting. The width of the prediction interval equals approximately 1.8 on a natural log scale (approximately 0.8 \log_{10}). Interactions (effects of) the variables *T:t*, *pH:t* and *pH:t^2* are not significant, but are part of the model, because *T:pH:t*, *pH:lnNa:t*, *T^2:t*, *pH^2:t* and *pH:lnNa:t* are (highly) significant. By convention, marginal effect terms are included in the model, if interactions are significant. Removing marginal effect terms from a fitted model is either statistically meaningless or futile in the sense that the model simply changes its parametrization to something equivalent (Venables and Ripley, 2002). Variable interactions *Ca:t*, *pH:lnNa:t* and *pH^2:day* were found to be the most significant variables determining μ_1 . Variable interactions *pH:t*, *lnNa:t* and *pH:lnNa:t* were found to be the significant variables determining μ_2 . These effects are visualised in Fig. 2 with the predictions of mean values of \log_{10} reductions of PRD1 concentrations after 200 days at 9.5 °C and after 20 days at 12 °C, including 95% prediction intervals, and of the values of μ_1 and μ_2 . Generally, the trends for \log_{10} reductions and μ_1 are similar, because μ_1 mostly determines the \log_{10} reductions of PRD1 concentrations. At 1 mM sodium concentration and without calcium, the \log_{10} reductions and μ_1 decrease stronger with increasing pH at 12 °C than at 9.5 °C. At 9.5 °C, inactivation is slower anyway. The value of μ_2 increases with increasing pH, independent of temperature.

By including variable *pH^2:t* in the joint log-square model, a non-linear relation between pH and inactivation could be described. Fig. 2 shows that $\log_{10}C_{200}/C_0$ and μ_1 at 9.5 °C are the lowest in the range of about pH 6.5 – pH 7.5, depending on sodium concentration. At higher sodium concentration inactivation is faster. At 1 mM sodium concentration, the value of μ_2 is almost independent of pH and about –0.00001. The value of μ_2 decreases with increasing sodium concentration and pH, which is consistent with the aforementioned observation that inactivation was stronger non-linear for a combined increase in pH and sodium concentration.

An increasing calcium concentration strongly increased the inactivation rate more than its contribution to an increase in IS only. Within the range of the experimental conditions, temperature is the most important factor determining inactivation, and the effect of pH is stronger at higher temperature (Fig. 2).

4. Discussion

In our study, for each experiment, estimates of μ_1 of the first-

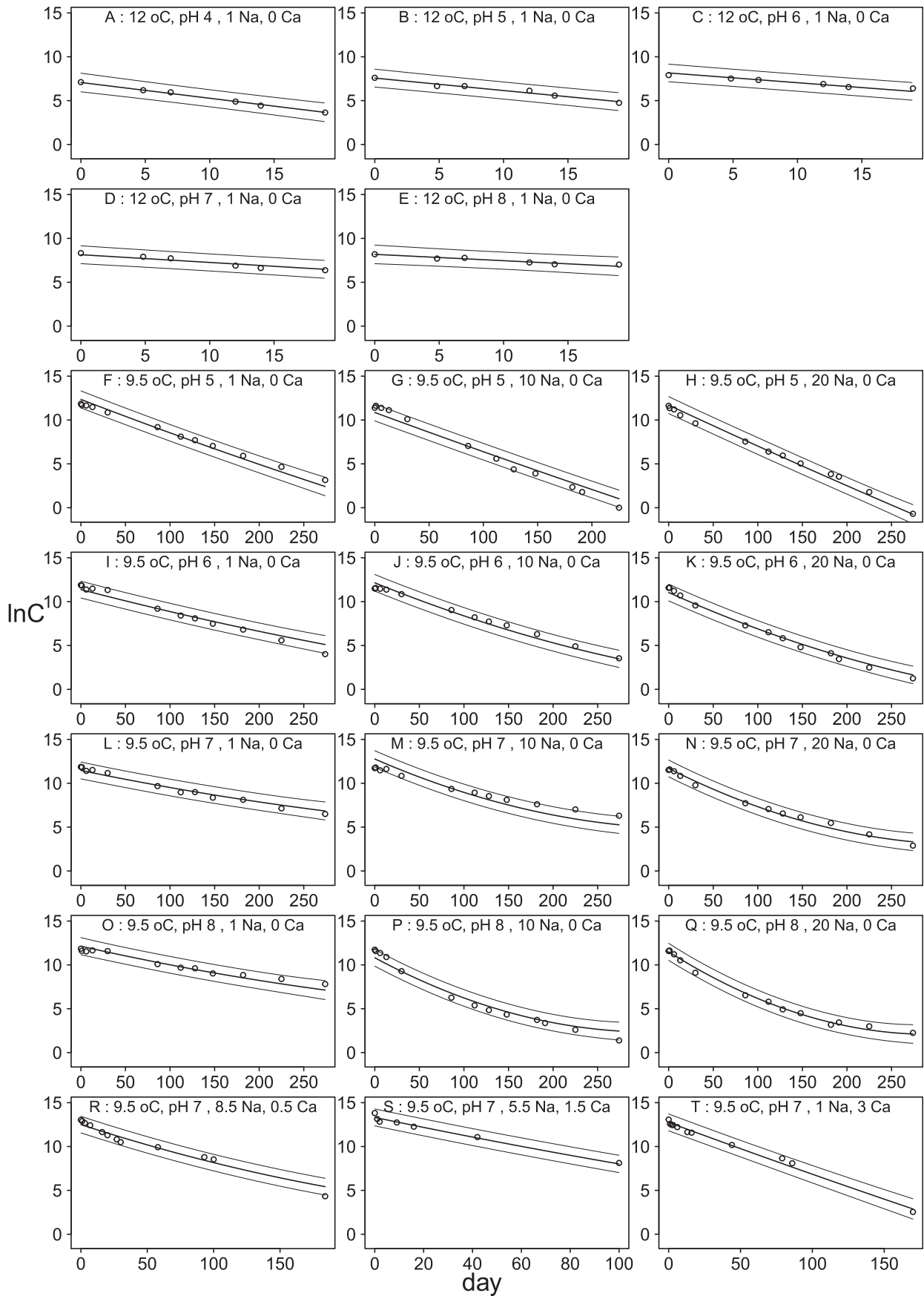


Fig. 1. PRD1-inactivation experiments A – T with conditions; open circles: measured concentrations; line: mean prediction with the joint log-square model; thin lines: 95% prediction interval.

Table 2
Coefficients of joint log-square model (Equations (4), (7) and (8)).

Coefficient	Dimension	Effect	Estimate	Std. Error	Probability
a_1	$^{\circ}\text{C}^{-1}\text{day}^{-1}$	$T:t$	-2.0×10^{-2}	1.9×10^{-2}	0.29
a_2	day^{-1}	$\text{pH}:t$	1.4×10^{-2}	3.7×10^{-2}	0.70
a_3	$(\ln\text{mM})^{-1}\text{day}^{-1}$	$\ln\text{Na}:t$	-1.0×10^{-2}	4.0×10^{-3}	0.00016
a_4	$\text{mM}^{-1}\text{day}^{-1}$	$\text{Ca}:t$	1.3×10^{-2}	9.5×10^{-4}	$<2 \times 10^{-16}$
a_5	$^{\circ}\text{C}^{-1}\text{day}^{-1}$	$T:\text{pH}:t$	-7.1×10^{-3}	3.8×10^{-3}	0.062
a_6	$(\ln\text{mM})^{-1}\text{day}^{-1}$	$\text{pH}:\ln\text{Na}:t$	3.7×10^{-3}	6.2×10^{-4}	1.7×10^{-8}
a_7	$^{\circ}\text{C}^{-2}\text{day}^{-1}$	$T^2:t$	4.5×10^{-3}	2.0×10^{-3}	0.023
a_8	day^{-1}	$\text{pH}^2:t$	3.7×10^{-3}	4.2×10^{-4}	9.2×10^{-16}
b_1	day^{-2}	$\text{pH}:t^2$	-2.1×10^{-6}	1.4×10^{-6}	0.12
b_2	$(\ln\text{mM})^{-1}\text{day}^{-2}$	$\ln\text{Na}:t^2$	5.4×10^{-5}	1.5×10^{-5}	0.00041
b_3	$(\ln\text{mM})^{-1}\text{day}^{-2}$	$\text{pH}:\ln\text{Na}:t^2$	-1.1×10^{-5}	2.3×10^{-6}	8.6×10^{-6}

T is temperature ($^{\circ}\text{C}$), t is time (day); Interactions of variables (effects) in R are designated by a ":"; Residual standard error $s = 0.46$; Multiple R-squared: 0.98.

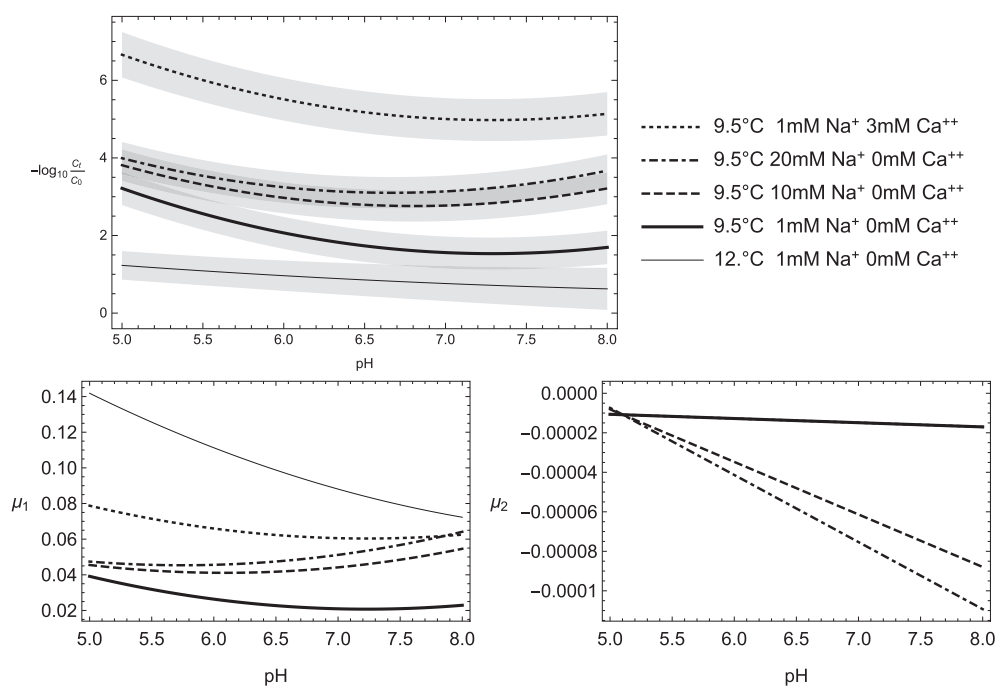


Fig. 2. Model behaviour: Mean log-square model predictions of PRD1 concentration reduction by inactivation after 200 days (9.5°C) and 20 days (12°C), including 95%-prediction intervals (gray areas), and of parameters μ_1 and μ_2 as a function of temperature, pH, sodium and calcium concentrations.

order rate model and of the log-square model are very similar. The estimates ranged between 0.066 day^{-1} and 0.19 day^{-1} for the 12°C experiments (A – E), between 0.020 day^{-1} and 0.051 day^{-1} for the 9.5°C experiments without calcium (F – Q) and between 0.044 day^{-1} and 0.058 day^{-1} for the 9.5°C experiments with calcium (R – T). Comparison with literature data on inactivation of PRD1 is not straightforward. John and Rose (2005) summarized literature data on inactivation of PRD1 over the temperature range of $7\text{--}23^{\circ}\text{C}$ from four studies and reported a μ_1 of 0.04 day^{-1} in a range of $0\text{--}0.3 \text{ day}^{-1}$. The equations of Bertrand et al. (2012) predict a value of 0.02 day^{-1} for PRD1 with a 95% prediction interval of $0.002\text{--}0.2 \text{ day}^{-1}$ at $9.5\text{--}12^{\circ}\text{C}$. This indicates that our estimates of the inactivation rate coefficient of PRD1 are well within the range reported in literature. The equations of Bertrand et al. (2012) are aimed at and limited to predicting the time needed for one \log_{10} reduction. In a simple matrix at 9.5°C , the prediction is 144 days with a 95% prediction interval of 12–1700 days and at 12°C , the prediction is 120 days with an interval of 10–1400 days. These are large prediction intervals.

The four studies (Blanc and Nasser, 1996; Dowd and Pillai, 1997;

Ryan et al., 2002; Yahya et al., 1993) referenced by John and Rose (2005) included wastewater effluent samples (Blanc and Nasser, 1996) and temperatures of $21\text{--}23^{\circ}\text{C}$ (Dowd and Pillai, 1997; Yahya et al., 1993).

The five studies on inactivation of PRD1 (Anders and Chrysikopoulos, 2006; Charles et al., 2008; Davies et al., 2006; Straub et al., 1992) referenced by Bertrand et al. (2012), also included sludge and soil as well as temperatures from 4 to 40°C . From those studies, estimates of a μ_1 at $4\text{--}9^{\circ}\text{C}$ are about 0.01 day^{-1} .

Our study did not focus on temperature and did not consider effects of organic matter as in wastewater. Although it is clear from literature that PRD1 is a persistent virus, temperature still determines its inactivation rate. At 12°C and above, temperature is by far the dominating factor. Even at the low temperature range of our experiments, temperature is the dominating factor determining the inactivation rate of PRD1, followed by calcium concentration, then by sodium concentration and finally by pH.

The choice of fitting the data jointly to the log-square model is because fitting to a two rate model gives numerical difficulties in fitting sums of exponentials, and, in addition, solutions may not be

unique (Stone et al., 2009). Indeed, with data from this study, such problems were encountered, and also fitting the joint data to the Weibull model was unsuccessful (not shown).

The drawback of the joint log-square model developed from experimental data at 9.5 °C and 12 °C is that it cannot reliably predict inactivation at other temperatures. The log-square model approximates both the two rate and Weibull models, but it is unsuitable for extrapolation (Stone et al., 2009). When extrapolating, for example for longer time periods, under conditions where inactivation proceeds non-linear, predictions of virus concentrations may start to increase in time. Despite this restriction, the log square model that we have developed covers the range of hydrochemical conditions encountered in Dutch groundwaters, whereby groundwater temperature is very much in the range of 9.5–12 °C (Sadeghi et al., 2011). Also considering the fact that PRD1 inactivation was monitored up to 274 days, there is little need for extrapolation.

It is common knowledge that salt concentration and pH affect the charged groups in proteins and, hence, may induce conformational changes. PRD1 has a stable outer protein coat and an inner lipid membrane connected by electrostatic interactions (San Martín et al., 2002). So, in the case of PRD1, these chemical conditions may affect the conformation of the coat proteins as well as the connection between coat and inner membrane and, consequently, the virus particle may not be infectious to its host bacterium anymore. The finding that the inactivation rate was lower between pH 6.5 – pH 7.5 and higher outside this range is qualitatively consistent with a study by Feng et al. (2003) who found that the inactivation rates of bacteriophages MS2 and Q β were lowest within the pH range 6–8. This may not apply in general to all viruses. Rossi and Aragno (1999) found no change in the inactivation rate of bacteriophage T7 in the pH range 5–9. At higher sodium concentrations, inactivation of PRD1 was faster. In the column experiments under the same range of conditions an increase of the attachment rate of PRD1 to sand grains due to higher sodium concentrations was observed (Sadeghi et al., 2011). This increase in attachment rate was attributed to the compression of double layers of ions reducing the intersurface potential energy according to DLVO theory. Double layer compression may also affect electrostatic interactions amongst functional groups in the protein coat and between the protein coat and inner membrane of PRD1 particles and render those particles inactive. In addition to inactivation, losses of countable virus particles may be due to attachment to the walls of the tubes in the experiments, or to other solid particles, or to aggregation of virus particles. Similarly, PRD1 appeared to inactivate faster with increasing calcium concentrations, which is in line with the study of Yates et al. (1985) where, in eleven groundwater samples, it was found that the inactivation rate of MS2 increased with calcium concentration. In our experiments, the effect of an increased calcium concentration was found to be stronger than just the contribution of calcium to IS. Mylon et al. (2009) studied MS2 stability in different mono- and divalent solutions including CaCl₂ in the range of 10–1000 mM. Their observations revealed that monovalent salts did not cause phage aggregation. Phage aggregation occurred with an increasing calcium concentration, but was not observed at values of Ca²⁺ less than 1 mM. Note that aggregation of virus particles leads to a reduction in the number of infectious particles, but at the same time may an aggregate of virus particles be a more stable infectious entity. To our knowledge, there is currently no theory to explain the effects of sodium and calcium concentration on virus inactivation.

5. Conclusions

An empirical formula was developed for predicting inactivation rate bacteriophage PRD1 as a function of temperature (narrow

range), pH, sodium, and calcium concentration in the ranges representative of most field conditions. The limitation of this log-square model is that it cannot be used for extrapolation beyond the experimental conditions. Also, it should be noted that the experimental setup of this study was not fully balanced. Within the experimental conditions not all combinations of temperature, pH, sodium and calcium concentrations were tested. Therefore, obviously, the model can be improved on the basis of experiments with a wider temperature range and more combinations of sodium and calcium concentrations. Investigation on effects of other electrolytes is also recommended. Commonly, filtration processes, such as attachment, as determined in small-scale column experiments cannot be easily extrapolated to the field scale, mainly due to all kinds of heterogeneities that occur in the field and that were not captured at smaller scale. However, the water phase itself is less complex and its pH and salt concentration are easy to measure.

Inactivation is a homogeneous reaction that is purely dependent on water composition and temperature. It is not affected by the presence of solid grains. In other words, the process is the same whether it occurs in water within the pore space or in a container. As such, it is independent of the spatial scale. So, we can assume that our observations on inactivation of bacteriophage PRD1 in water in batch experiments are valid for groundwater. That is why one may be confident that these inactivation rates can be applied to the field situation under the studied physico-chemical conditions. Also, because long-term inactivation was considered, the developed log-square model may be applied at field scale for predicting inactivation during subsurface transport of virus within the range of the studied conditions.

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