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Inactivation of bacteriophage MS2 upon exposure to very low concentrations of chlorine dioxide

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ABSTRACT

This study investigates the effects of very low concentrations of ClO_2 applied in drinking water practice on the inactivation of bacteriophage MS2. Concentrations of 0.5 mg/L, 0.1 mg/L and 0.02 mg/L ClO_2 inactivated at least 5 log units of MS2 after an exposure time of approximately 20, 50 and 300 min respectively. When the ClO_2 concentration was as low as 0.005 mg/L, inactivation of 1 log unit MS2 was observed after 300 min exposure. Increasing the contact time to 24 h did not increase the inactivation any further. Non-linear inactivation kinetics (tailing) were observed for all conditions tested. Repeated addition of MS2 to the reactor showed that tailing was not caused by a reduction of the biocidal effect of ClO_2 during disinfection. The Modified Chick-Watson, the Efficiency Factor Hom (EFH) model and the Modified Cerf model, a modification of the two-fraction Cerf model, were fitted to the non-linear inactivation curves. Both the EFH and the modified Cerf model did fit accurately to the inactivation data of all experiments. The good fit of the Modified Cerf model supports the hypothesis of the presence of two subpopulations. Our study showed that ClO_2 is an effective disinfectant against model organism MS2, also at the low concentrations applied in water treatment practice. The inactivation kinetics followed a biphasic pattern due to the presence of a more ClO_2-resistant subpopulation of MS2 phages, either caused by population heterogeneity or aggregation/adhesion of MS2.

1. Introduction

Pathogenic enteric viruses originating from human excreta are frequently present in surface water, and because of their particle stability they can persist in this environment for weeks to months (de Roda Husman et al., 2009). Despite the application of sophisticated water treatment strategies for drinking water production, these viruses remain important sources of waterborne outbreaks and therefore a concern for human health (Carter, 2005; Lodder and de Roda Husman, 2005).

In order to avoid drinking water related outbreaks, the US Environmental Protection Agency (EPA) has launched the Long Term 2 Enhanced Surface Water Treatment Rule for the US (USEPA 2006), which requires a removal or inactivation of enteric viruses from source water by at least 4 logs (99.99%). In the Netherlands, regulations require a risk assessment to show that the risk of drinking water related infections is below 1 infection per 10,000 consumers per year (Anonymous, Dutch Drinking Water legislation, 2001).

Surface water treatment generally includes mechanical purification processes, followed by a disinfection treatment to kill residual micro-organisms. Worldwide, primarily chlorine disinfection is used to achieve the required reduction of pathogenic organisms, but chlorine has negative side effects like the formation of harmful disinfection by-products (DPB) like trihalomethanes (Rook, 1974). Alternatives like chlorine

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dioxide (ClO_2) have been proposed to prevent DPB formation. Advantages of ClO_2 disinfection include; no formation of trihalomethanes, oxidation of iron and manganese and it is a relative persistent residual. Disadvantages include formation of chlorite and chlorate and organic halides (Aieta and Berg, 1986). ClO_2 is sporadically used as primary disinfectant, but at some locations in Europe ClO_2 is added to finished water to maintain a disinfectant residual in the distribution network to prevent regrowth and provide protection against ingress of fecal contaminants. Under these conditions, very low concentrations of ClO_2, generally not exceeding 0.1 mg/L are added to the finished water.

ClO_2 effectively inactivates micro-organisms in water and inactivation of several water related pathogenic viruses has been reported (Alvarez and O'Brien, 1982; Chen and Vaughn, 1990; Thurston-Enriquez et al., 2005; Berman and Hoff, 1984; Lim et al., 2010b). Most of these studies used relative high concentrations of ClO_2 in combination with short contact times, but in water applications frequently low concentrations are dosed, and inactivation is achieved by long contact time.

The level of disinfection is expressed as *Ct* values, which is the disinfectant concentration (in mg/L) multiplied by the contact time (in minutes). The Chick-Watson model, describing first order inactivation kinetics is routinely used to calculate *Ct* values necessary to achieve the required level of disinfection. Based on previous viral disinfection studies the EPA has listed minimal *Ct* values for inactivation of viruses (USEPA, 2006). However, many observations have shown that viral inactivation kinetics using disinfectants does not follow first order kinetics, but shows significant tailing. Several explanations for tailing in general have been proposed (Cerf, 1977; Xiong et al., 1999) including (i) aggregation of viruses or attachment of viruses to particles in water, herewith protecting the target organism against exposure to the disinfectant (Thurston-Enriquez, 2003; Keswick et al., 1985; Berman and Hoff, 1984) (ii) the presence of subpopulations of the target organism with differences in resistance against the disinfectant (Hiatt, 1964; Gerba et al., 2003) (iii) a decreasing biocidal effect of the inactivating agent during the disinfection treatment (Hiatt, 1964). These non-linear inactivation curves are not adequately described by the Chick-Watson model. Other models including the Efficiency Factor Hom (EFH) model have been developed to describe non-linear inactivation curves more accurately (Gyürék and Finch, 1998; Xiong et al., 1999; Haas and Joffe, 1994). These models describe non-linear inactivation fairly precise. However, none of these models reveal information about biological causes of non-linear viral inactivation kinetics.

The current study investigates the inactivation of viruses by ClO_2, using bacteriophage MS2 as model organism for human enteric viruses. MS2 is frequently used either as indicator organism or as model organism for enteric viruses, because it exhibits similar morphology and is relatively resistant to disinfection treatments compared to human pathogenic viruses (Sobsey, 1989, IAWPRC Study Group, 1991). It functions therefore as an appropriate candidate representing human enteric viruses present in the environment.

The objectives of this study were to (i) determine the inactivation of MS2 at the low concentrations of ClO_2 applied in drinking water practice (ii) to determine the lower threshold of ClO_2 concentrations able to inactivate MS2 (iii) investigate if the *Ct* concept is valid for very low concentrations of ClO_2 and (iv) investigate the cause of tailing observed during inactivation of MS2.

2. Materials and methods

2.1. Virus strain and assay

The bacteriophage MS2 solution containing 1×10^{12} pfu/ml was obtained from GAP Enviromicrobial Services (London, Canada). Purification by GAP Enviromicrobial Services included centrifugation followed by filtration of MS2 using a 0.2 μm filter to remove particles and cell debris. The double layer agar method was used to enumerate MS2 after the disinfection treatments according (ISO 10705-1) using *Salmonella typhimurium* WG49 as host organism (ISO, 1995). All experiments were performed in duplicate.

2.2. ClO_2 production and measurement

ClO_2 was produced using a Halox H1000SRE unit (Halox technologies, Bridgeport, CT, USA). A ClO_2 solution with a concentration of approximately 2.0 g/L was produced and diluted to 800 mg/L ClO_2 stock solution. This stock solution was stored at 4 °C in the dark and remained stable for at least half a year. For disinfection experiments, the stock solution was further diluted to a working stock solution with concentration between 1 and 5 mg/L. The ClO_2 concentration of the working stock was determined by measuring the absorbance directly at 359 nm (the molar absorptivity is 1106 L/mol cm at 359 nm) in a 1 cm quartz cuvette. ClO_2 concentrations during disinfection experiments with a concentration between 0.7 mg/L and 0.2 mg/L ClO_2 were determined by measuring the absorbance at 359 nm, using a 5 cm quartz cuvette. Below 0.2 mg/L ClO_2 this method is not sufficiently accurate, and therefore ClO_2 concentrations with a concentration between 0.2 and 0.05 mg/L were determined by using the spectrophotometric method measuring the discoloration of the indicator chlorophenol red, as described by Fletcher and Hemmings (1985). This method allows accurate determination of ClO_2 concentrations from 0.05 mg/L and higher. Samples were measured at 575 nm using 5 cm cuvettes for maximum sensitivity. Buffered demand free water was used as blanco.

2.3. Experimental conditions

All glassware was made ClO_2 demand free by overnight soaking in 5 mg/L of ClO_2, followed by thorough rinsing with ultrapure MQ (Millipore, Massachusetts, US) water before use. Disinfection experiments were performed in two batch reactors (1000 ml erlenmeyers), each filled with 900 ml buffered demand free water (BDF). BDF water was made by addition of 10 ml. 0.5 M sodium phosphate buffer pH 7.2–990 ml of ultrapure MQ water, resulting in 5 mM sodium phosphate buffered demand free water, pH 7.2. The reactors were kept on ice and continuously stirred to keep the temperature at 0 °C. Reactor 1 was used for disinfection experiments and before starting 100 ml of a 10× ClO_2 stock solution of the appropriate concentration was added. After mixing, samples (50 ml in

duplicate) were taken to determine the concentration of ClO_2 in the reactor, resulting in 900 ml ClO_2 solution in the reactor. Subsequently 1 ml of MS2 stock consisting of $\pm 1 \times 10^{10}$ PFU ml was added ($t = 0$) to the reactor resulting in a concentration of approximately 1×10^7 PFU ml infectious MS2 in the reactor. To determine the number of remaining infectious MS2 phages during disinfection, 5 ml samples were taken at appropriate time points, and immediately quenched in 0.5 ml 1% (w/v) sodium thiosulfate and stored on ice. ClO_2 quenched with sodium thiosulfate did not show any inactivation of MS2 (data not shown). Furthermore samples were taken from reactor 1 during the experiment to determine the ClO_2 decay. Reactor 2 was used to determine the initial concentration of MS2 at $t = 0$ by addition of 1 ml of MS2 stock consisting of 1×10^{10} PFU ml to 900 ml BDF water. BDF water (ultrapure MQ water with 5 mM sodium phosphate pH 7.2) demonstrated negligible ClO_2 demand.

Additionally ClO_2 disinfection experiments were done in quadruplicate with initial ClO_2 concentration of 0.5, 0.25, 0.1 and 0.05 mg/L respectively, to determine the effect of the initial ClO_2 concentration on the ClO_2 decay rate. For this, ClO_2 decay was followed for at least 120 min disinfection time, and the ClO_2 concentration was measured at minimally 5 time points for each disinfection experiment.

2.4. Kinetic modeling

The Solver function in Microsoft Excel 2003 was used to minimize the sum of squares of the difference between the measured ClO_2 concentration and calculated result of first order kinetic equation (equation (1)), to determine the ClO_2 decay constant (k').

$$C = C_0 \exp(-k't) \quad (1)$$

where C and C_0 are the ClO_2 concentration (mg/L) at time t (min). k' is the first order disinfectant decay rate constant per minute (Haas and Joffe, 1994).

The inactivation kinetics of MS2 by ClO_2 were described using the modified Chick-Watson equation (3), which is derived from the classical Chick-Watson model (2) but includes a factor for disinfectant decay.

$$\text{Chick} - \text{Watson} \quad \ln N/N_0 = -kCt \quad (2)$$

$$\text{Modified Chick} - \text{Watson} \quad \ln N/N_0 = -kC_0^n/nk' \times \left[1 - \exp(-nk't)\right] \quad (3)$$

where $\ln N/N_0$ is the natural log of the survival ratio (=number of infectious MS2 phages at time t divided by the number at $t = 0$), k is the inactivation rate constant of the target organism (MS2) and n is the coefficient of dilution which represents the average number of molecules combined with the organism necessary to cause inactivation (Gyürék and Finch, 1998).

The Efficiency Factor Hom (EFH) model is frequently applied to calculate Ct values for non-linear inactivation curves (Thurston-Enriquez et al., 2003, 2005; Lim et al., 2010a), and can be considered as an acceptable approximation of the Hom model for systems following first order disinfectant decay and showing non-linear inactivation kinetics (Haas and Joffe, 1994).

$$\text{EFH} \quad \ln N/N_0 = -kC_0^n t^m \left[\left(1 - e^{\{-nk't/m\}}\right) \Big/ \left(-nk't/m\right)\right]^m \quad (4)$$

where m is Hom's exponent.

To obtain values for k, n and m, Excel solver was used to minimize the sum of squares of the difference between the observed and calculated $\ln N/N_0$ for each disinfection experiment separately. Data from single experiments were used to estimate the model parameters for all models.

3. Results and discussion

3.1. Inactivation of MS2 with low concentrations of ClO_2

3.1.1. ClO_2 concentration during disinfection

Disinfection experiments were conducted with ClO_2 concentrations of approximately 0.5 mg/L, 0.1 mg/L, 0.02 mg/L and 0.005 mg/L. For disinfection experiments with an initial ClO_2 concentration of 0.02 mg/L and 0.005 mg/L, the ClO_2 concentration and ClO_2 decay in the reactor could not be determined due to the detection limit of 0.05 mg/L of the chlorophenol red method. Therefore the initial ClO_2 concentration of these experiments was determined by measuring the concentration of the 10× working stock shortly before addition to the reactor. As to our knowledge no information exists about ClO_2 decay at very low concentrations, we had to estimate a k' value for these experiments. To make this estimation as accurate as possible, ClO_2 decay was measured over time for 15 individual disinfection experiments with various initial ClO_2 concentrations ranging from 0.6 mg/L to 0.05 mg/L ClO_2,and the first order disinfectant decay rate (k') was derived (Table 1). This should reveal any relationship between the initial ClO_2

Table 1 – Initial ClO_2 concentrations and ClO_2 decay constant k' for 15 disinfection experiments with initial concentrations between approximately 0.6 and 0.05 mg/L ClO_2. Disinfection decay calculations were based on at least 5 data points for each individual experiment.

	ClO_2 concentration	
	Measured	
Exp	ClO_2[a]	k' (min^{-1})
1	0.53	0.00072
2	0.57	0.00086
3	0.62	0.00098
4	0.63	0.00131
5	0.25	0.00107
6	0.28	0.00119
7	0.29	0.00126
8	0.094	0.00086
9	0.094	0.00092
10	0.116	0.00119
11	0.099	0.00150
12	0.033	0.00107
13	0.047	0.00136
14	0.056	0.00175
15	0.042	0.00171

a Concentration in mg/L.

concentration and k' in this concentration range, and extrapolation to lower ClO_2 concentrations may represent the best approximation of k' at very low concentrations. To get better insight in the relationship between the decay rate and the initial concentration, k' versus initial concentration was plotted for each individual experiment (Fig. 1). No obvious increasing or decreasing dependence of k' to the initial ClO_2 concentration could be revealed. A Pearson's correlation showed that no significant trendline could be derived from the average plotted data points ($p = 0.085$). Hence, we assume that the ClO_2 decay rate is independent from the initial ClO_2 concentration within the concentration range considered. With this information, it was decided to use the average decay rate of 0.001183 ± 0.000304 for all disinfection experiments, and this number was considered describing the decay for all concentrations accurately, including the ClO_2 decay for disinfection reactions with ClO_2 concentrations below the detection limit. To determine the sensitivity of the models for variation in k', the 2.5 percentile and 97.5 percentile from the obtained k' values were determined at 0.00077 and 0.00173. These values were used to determine the sensitivity of the models for deviations from the average k'.

3.1.2. *Inactivation of MS2*

Disinfection experiments with initial concentrations of 0.39 and 0.46 mg/L ClO_2 were able to reduce the number of infectious MS2 with at least 5 log units in 20 min (Fig. 2). Disinfection with an initial ClO_2 concentration of 0.10 mg/L required an exposure time of 50 min for 5 log unit reduction of MS2 phages. Also an initial concentration of 0.02 mg/L ClO_2 was able to reduce the number of infectious MS2 with 5 log, although 300 min of contact time was required for this reduction. The residual ClO_2 after 300 min could be calculated using the k' derived as described in Section 3.1.1 and was 0.013 and 0.014 mg/L for the two experiments. In order to further reduce the initial amount of ClO_2, a disinfection experiment with a very low initial concentration of 0.005 mg/L ClO_2 was done. This concentration did reduce the infectious MS2 population, but with only 1 log unit in approximately 300 min.

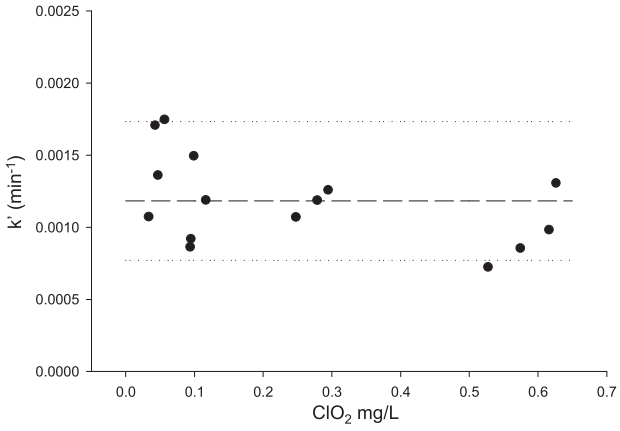

Fig. 1 – A scatter plot showing the ClO_2 decay rate (k') versus the initial ClO_2 concentration for 15 disinfection experiments. The average k' value of 0.001183 is shown by a dashed line. The dotted lines show the 2.5 percentile and 97.5 percentile values of the obtained k' values.

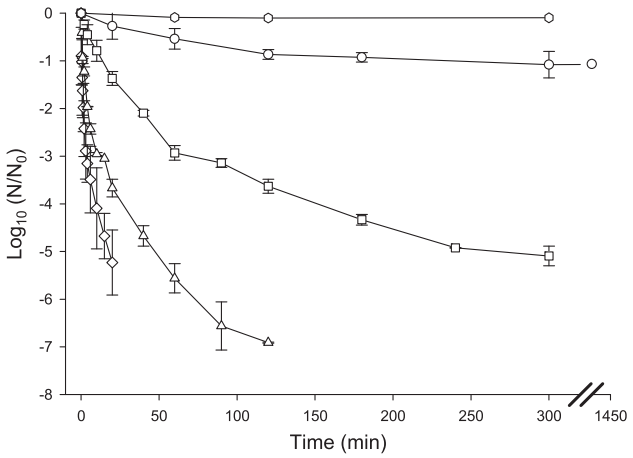

Fig. 2 – Inactivation of MS2 after exposure to 0.43 (diamonds), 0.1 (triangles), 0.02 (squares), 0.005 (circles) and 0 (hexagons) mg/L ClO_2. The inactivation curves show average inactivation of duplicate disinfection experiments. Note the break on the *x*-axis to show inactivation after 1440 min contact time.

Prolongation of the contact time to 24 h did not reduce the infectious population any further (Fig. 2). These results show that an initial concentration of 0.005 mg/L ClO_2 is capable of reducing the MS2 population by 1 log unit in approximately 300 min, but then inactivation ends. Inactivation may have stopped either by disappearance of the total ClO_2 amount after 300 min, or by reaching a ClO_2 concentration that is not capable of inactivating MS2 anymore. Because it is not possible to measure these low residual ClO_2 concentrations, none of these possibilities can be excluded. However, the *Ct* concept states that "the level of inactivation can be assigned to a given *Ct* value, independently of the disinfectant concentration used". The inactivation curves versus *Ct* (mg/L min) of the disinfection experiments in this study (composed by various combinations of initial ClO_2 concentrations multiplied by time) confirm this concept (Fig. 3). Assuming that the disinfectant rate constant k' is concentration independent for very low ClO_2 concentrations, the calculated ClO_2 residual in the reactor after 300 min disinfection is 0.0034 mg/L, and 0.00089 mg/L after 24 h (1440 min). Exposure to a ClO_2 concentration of 0.0034 mg/L for 1140 min (1440–300 min) including accounting for ClO_2 decay results in an additional 1.83 *Ct* (mg/L min) for the 1140 min timeframe (Fig. 4). It can be derived from Fig. 3 that 1.83 *Ct* results in an inactivation of approximately 3 log, but no inactivation is observed. This suggests that, assuming the *Ct* concept is valid for low concentrations, inactivation ended after the 300 min time point because ClO_2 had disappeared from the reactor by decay or volatilization.

3.2. *Non-linear inactivation of MS2*

Tailing is frequently observed in disinfection experiments of water related pathogenic viruses (Finch and Fairbairn, 1991; Simonet and Gantzer, 2006; Alvarez and O'Brien, 1982). The

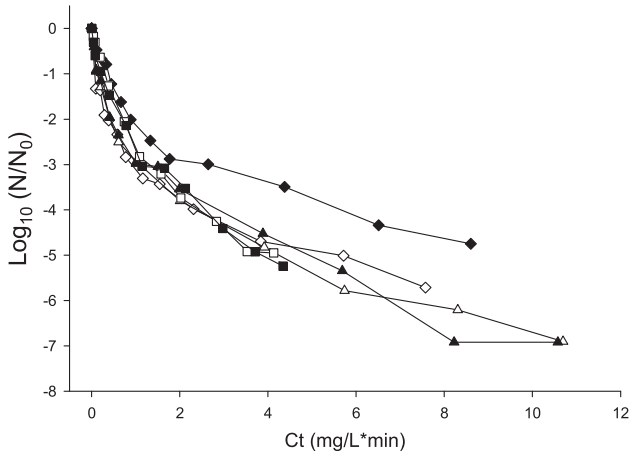

Fig. 3 – Inactivation of MS2 after exposure to 0.5 (diamonds), 0.1 (triangles) and 0.02 mg/L (squares) ClO_2, in which inactivation is being displayed versus *Ct* (mg/L min). First experiment (open symbols), duplo experiment (closed symbols).

inactivation curves obtained from the disinfection experiments described in the current study showed non-linear inactivation for all ClO_2 concentrations applied. MS2 inactivation occurred relatively fast directly after initiation of the disinfection treatment, and slowed down when the treatment proceeded. Rapid inactivation occurred for about 3 log inactivation, followed by continued inactivation but at a lower rate. This resulted in non-linear inactivation curves, showing tailing or a biphasic shape (Figs. 2 and 3). This could be attributed to subpopulations within the MS2 phage population caused by attachment to particles or intrinsic heterogeneity of the population, or to disinfection reaction conditions that alter during the disinfection time and herewith cause a reduction of the biocidal effect of ClO_2. To exclude this latter possibility as a cause of tailing for the experiments of this study, the biocidal effect of ClO_2 was determined during the time of the disinfection experiment.

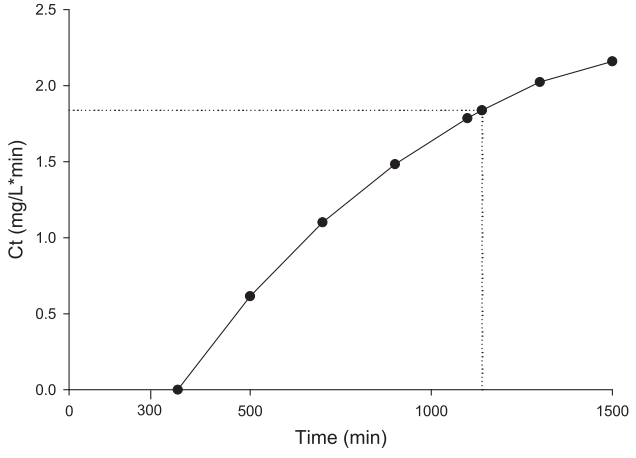

Fig. 4 – Cumulative *Ct* credits obtained after exposure for 1500 min, starting with an initial ClO_2 concentration of 0.0034 mg/L ClO_2 at 300 min. This concentration represents the calculated concentration present after 300 min disinfection with an initial concentration of 0.005 mg/L ClO_2 at 0 min. The 1140 min time point reflects 24 h disinfection, resulting in an additional *Ct* credit of 1.83 *Ct*, as is shown by the dotted lines.

3.3. *Changes in biocidal effect of ClO_2 during disinfection*

A possible decline of the biocidal effect of ClO_2 during the disinfection experiments was investigated, using an experimental setup similar to the previous disinfection experiments. Considering the disinfection experiment with a concentration of 0.10 mg/L ClO_2, the transfer from fast to slow inactivation occurred after approximately 10 min (Fig. 7). If the reduction in inactivation rate is caused by a diminished biocidal effect of ClO_2 after approximately 10 min disinfection, fresh MS2 phages added to the disinfection reactor after 10 min disinfection are expected to follow slow inactivation kinetics. Addition of fresh MS2 after 10 min reaction time did not result in a low inactivation rate of the added population (Fig. 5). Their inactivation was similar to inactivation of the MS2 population added at $t = 0$. Addition of fresh MS2 after 60 min again showed similar inactivation kinetics (data from single experiment not shown). These results indicate that the biocidal effect of ClO_2 did not diminish during disinfection, and was identical to the situation at $t = 0$. Similar results regarding the biocidal effect of ClO_2 have been observed for ClO_2 disinfection of bacterial subpopulations (Berg et al., 1988).

As the inactivation conditions apparently did not change during disinfection, tailing could be caused by intrinsic resistance differences of individual MS2 phages. In that case MS2 phages representing the slow phase of inactivation (the tail) are more resistant against ClO_2 and exposure to fresh ClO_2 would not result in faster inactivation. To determine this, the population representing the tail was transferred to a freshly prepared reactor containing BDF with 0.1 mg/L ClO_2. This population of more ClO_2-resistant phages was obtained by disinfection of 1×10^9 PFU of the normal MS2 population for 10 min in 0.1 mg/L ClO_2, resulting in approximately 3 log

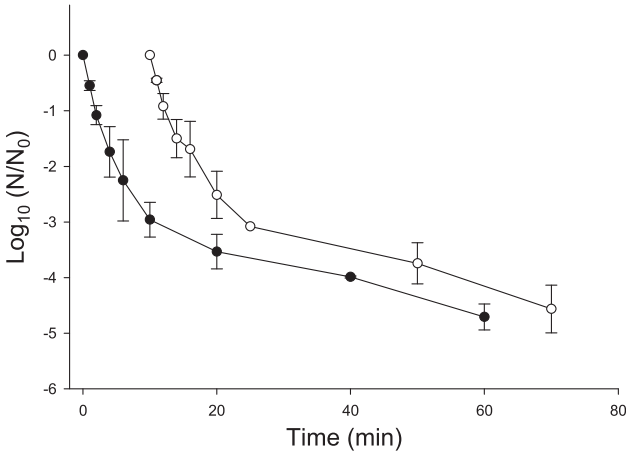

Fig. 5 – Inactivation curve of an MS2 population added at $t = 0$ (closed symbols) to the reactor, and inactivation of a similar MS2 population added to the same reactor after 10 min of disinfection (open symbols). Both populations show similar inactivation kinetics.

inactivation of the ClO_2-sensitive MS2 population. 100 ml of the resulting reaction mixture containing approximately 1×10^6 PFU of the ClO_2-"resistant" MS2 population was immediately transferred to a batch reactor containing 900 ml fresh 0.1 mg/L ClO_2. Fig. 6 shows that disinfection of this population resembles the slow inactivation at the tail of the non-pretreated population. These results exclude the possibility of diminishing biocidal effect of ClO_2 during disinfection, and therefore the variance in inactivation kinetics is a property of the MS2 phage population.

3.4. *The resistant subpopulation representing the tail*

The current study shows that tailing is a characteristic of the MS2 population and the population consisted of at least two subpopulations that differ substantially in their resistance against ClO_2. The majority of the population (approximately 99.9%) consisted of MS2 that was rapidly inactivated, while approximately 0.1% of the population required prolonged exposure to ClO_2 for inactivation. Additional resistance may be an intrinsic property of a heterogeneous virus population, or can be acquired later e.g. by aggregation with other virus particles or by attachment or adhesion to particles in the environment.

Several studies have concluded that adhesion to particles or aggregation formation provides protection to viruses, herewith reducing disinfection efficacy and probably introducing tailing (Berman and Hoff, 1984; Thurston-Enriquez et al., 2003; Urakami et al., 2007). The conditions in the current study used BDF water and were performed under laboratory conditions, so attaining additional resistance by particle attachment is unlikely. Furthermore, experimental conditions do not favor aggregation of MS2 as the low isoelectric point of MS2 of 3.9 results in repulsion rather than attraction at the pH used during the disinfection experiments (Gerba, 1984). However, we cannot experimentally exclude aggregation or adhesion to particles. Also intrinsic variation in the phage population, resulting in phages with increased resistance might be an important source of tailing. Synthesis of MS2 in the host is a self-regulating assembly process and it is not unlikely that this process results in virions that individually differ from each other. For example variation might exist in the structure of the capsid, resulting in variance in resistance against disinfectants.

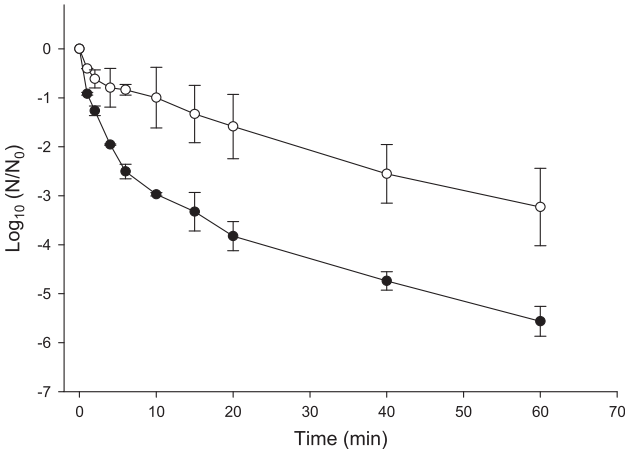

Fig. 6 – Comparison of the inactivation kinetics of MS2 phages representing the tail of the inactivation curve (open symbols) with inactivation kinetics of the initial population (closed symbols).

3.5. *The shape of the inactivation curve*

The shape of the inactivation curves observed can be described as upward concave or biphasic (Fig. 2, 3 and 5). A variety of disinfection models have been described for these types of inactivation curves (Gyürék and Finch, 1998; Xiong et al., 1999). These mostly empirical models were designed to properly determine *Ct* values based on inactivation experiments of waterborne pathogens or model organisms. Because of its simplicity, the Chick-Watson model (equation (1)) is predominantly used for *Ct* calculations in drinking water regulatory practice, though it has been described that it does not predict non-linear inactivation curves very precise (Gyürék and Finch, 1998). The EFH (equation (4)) describes non-linear inactivation curves more adequately, by adapting the parameter m in combination with contact time. Variance of m gives more or less importance to contact time, with $m < 1$ describing tailing of the inactivation curve. The EFH model empirically describes inactivation curves, but does not reveal much about biological phenomena involved in tailing.

For all disinfection experiments, the inactivation rate constant k was determined using the MCW model, with $n = 1$ to come close to the traditional Chick-Watson model. For the EFH model k, n and m where determined (Table 2) based on the best fit determined by minimizing the sum of squares. Because these models include disinfectant concentration C multiplied by contact time t, the decrease of disinfectant concentration should be compensated for by an increase in time. As concentration was the only variable in the disinfection experiments described, and inactivation versus *Ct* showed similar inactivation for all combinations of ClO_2 concentration versus time (Fig. 3), the outcome of the EFH model parameters are expected to be close to equal for each concentration. However, variation is found for the parameters n and m in case of the EFH model. This indicates that EFH model may not accurately predict inactivation of MS2 when extrapolated to other ClO_2 concentrations/contact time combinations than what was used in the disinfection experiments described.

Since our results suggested population heterogeneity as main cause for tailing, a biphasic inactivation curve was considered. A typical biphasic inactivation curve results when the population of the target organism consists of two subpopulations, each with a different resistance to disinfectants (Cerf, 1977). Cerf derived a mechanistic two-fraction model that specifically describes the inactivation of two populations with differing inactivation kinetics. Cerf's model has not been used before in the field of water treatment, and is predominantly used to describe the existence of two subpopulations with different resistance characteristics against food preservation treatments (predominantly thermal treatments). This model does not contain a parameter for disinfectant concentration. Because the experiments in this study focused on low concentrations of ClO_2 in combination with long contact times, ClO_2 decay during disinfection time

Table 2 – Summary of parameter estimates for the Modified Chick-Watson model, the EFH model and the modified Cerf model, and ESS and R^2 values for comparison of predicted and observed ClO_2 inactivation curves. Raw data were used for estimation of the model parameters. The ESS and R^2 values between brackets were calculated using the Cerf model without accounting for disinfectant decay.

Modified Chick-Watson (MCW model)

Exp	ClO_2 mg/L	Data points[b]	k	n	ESS[c]	R^2
1a	0.39	12	2.245	1	166.942	0.861
1b	0.45	12	1.581	1	70.857	0.851
2a	0.10	12	1.731	1	142.693	0.880
2b	0.10	12	1.951	1	144.854	0.885
3a	0.019	11	2.903	1	41.785	0.886
3b	0.020	11	2.825	1	44.670	0.913
4a	0.0049	5	2.095	1	0.953	0.817
4b	0.0051	5	2.709	1	1.951	0.849

EFH

Exp	ClO_2 mg/L	Data points[b]	k	n	m	ESS[b]	R^2
1a	0.39	12	9.771	0.732	0.335	1.516	0.988
1b	0.45	12	5.978	0.827	0.438	3.754	0.970
2a	0.10	12	6.441	0.372	0.375	1.763	0.992
2b	0.10	12	6.316	0.416	0.405	2.976	0.987
3a	0.019	11	15.249	0.800	0.537	3.187	0.984
3b	0.020	11	6.659	0.544	0.496	1.557	0.991
4a	0.0049	5	5.002	0.757	0.593	0.384	0.912
4b	0.0051	5	2.663	0.400	0.396	0.025	0.995

Modified Cerf

Exp	ClO_2 mg/L	Data points[b]	f	$k1$	$k2$	ESS[c]	ESS[c]	R^2	R^2
1a	0.39	12	0.9978	14.687	0.977	6.638	(6.698)	0.954	(0.953)
1b	0.45	12	0.9942	5.928	0.679	0.649	(0.656)	0.995	(0.995)
2a	0.10	12	0.9991	10.939	0.839	5.822	(6.380)	0.976	(0.974)
2b	0.10	12	0.9976	11.434	0.984	5.873	(6.537)	0.981	(0.976)
3a	0.019	11	0.9962	6.759	1.282	0.924	(1.186)	0.995	(0.994)
3b	0.020	11	0.9905	9.019	1.513	1.994	(2.379)	0.990	(0.989)
4a	0.0049	5	nd[a]						
4b	0.0051	5	nd[a]						

a Not determined because inactivation is not biphasic.
b Number of data points used for calculation of the inactivation curve.
c Error sum of squares.

should preferably not be ignored. Therefore disinfectant concentration and decay was implemented in the Cerf model.

$$\text{CERFs model} \quad N(t)/N_0 = f e^{(-k1\times t)} + (1-f)e^{(-k2\times t)} \tag{5}$$

in which $N(t)/N_0$ represents the survival ratio (=number of infectious MS2 phages at time t divided by the number at $t = 0$), $k1$ and $k2$ are the inactivation rate constants for population 1 and 2 respectively, and f is the initial proportion in the less resistant fraction. Expressed in natural logarithm this can be rewritten as:

$$\ln(N(t)/N_0) = \ln\left(f e^{(-k1\times t)} + (1-f)e^{(-k2\times t)}\right)$$

To adapt this model for chemical inactivation, we replaced the constant t for Ct (concentration multiplied by time) which results in:

$$\ln(N(t)/N_0) = \ln\left(f e^{(-k1Ct)} + (1-f)e^{(-k2Ct)}\right)$$

This formula describes the inactivation of two independent subpopulations both following first order kinetics, and factors $-k1Ct$ and $-k2Ct$ are similar to Chick-Watsons model describing first order inactivation (equation (2)). Modification of the Chick-Watsons model for inactivation conditions under disinfectant demand conditions resulted in the MCW model (equation (3)). Implementation of MCW in Cerf's model by replacing $-kCt$ with $-kC_0^n/nk' \times [1 - e(-nk't)]$ results in a modified Cerf model capable of describing two-fraction inactivation under disinfectant decay following first order kinetics:

$$\ln N(t)/N_0 = \ln f e\left(-k1C_0^n/nk' \times \left[1 - e(-nk't)\right]\right) + (1-f)e \left(-k2C_0^n/nk' \times \left[1 - \exp(-nk't)\right]\right) \tag{6}$$

For this modified Cerf model, the model parameters f, $k1$ and $k2$ were determined with $n = 1$. For individual disinfection experiments the best fit was determined by minimizing the sum of squares between the observed and the calculated value. Both the Modified Cerf model and the EFH model produced an accurate fit to the data (Table 2). Based on the Error sum of squares (ESS) and correlation coefficients (R^2), the EFH model produced a slightly better fit to the data compared to the Cerf model. Comparison of the results of the Cerf model

(without disinfectant decay) with the Modified Cerf model (including disinfectant decay) showed that the Modified Cerf model improved the fit of the model for each experiment (see ESS and correlation coefficient in Table 2). The fit of the MCW, EFH and modified Cerf model on the inactivation data of 0.39, 0.45, 0.1, 0.1, 0.019 and 0.020 mg/L ClO_2 are shown in Fig. 7.

To summarize, the modified Chick-Watson cannot describe the experimental data very well, as it cannot describe deviations from linear inactivation ($n = 1$). Both the empirical EFH model and the mechanistic Modified Cerf model are well capable of describing the inactivation curves for all individual disinfection experiments. The EFH model produced the most accurate fit based on the sum of squares and correlation coefficient. However, the accurate fit of the modified Cerf model originally designed to describe inactivation of two subpopulations, suggests the presence of two subpopulations of MS2 which differ en their resistance against ClO_2.

To find out how sensitive the models are for variations in the ClO_2 decay rate k' the 2.5 percentile and 97.5 percentile as determined in Section 3.1.1 of 0.00077 and 0.00173, were transferred to the Chick-Watson, the EFH and Modified Cerf model. Using these values did change the inactivation curves minimally for the disinfection experiments as was determined by comparing R^2 of confidence interval values with R^2 of the average k' value. For the Chick-Watson model, the maximal deviation from the R^2 obtained with the average k' value was 2.3%, specifically for the experiments with disinfection times of 300 min. The maximal deviation from R^2 found for the EFH model using the values of the 95% confidence interval was below 0.8%, while incorporation of 95% confidence interval values of k' in the Modified Cerf model only showed <0.1% deviation from the R^2 obtained from the average k' value. The modified Cerf model showed to be highly unsensitive for the observed variation in k'.

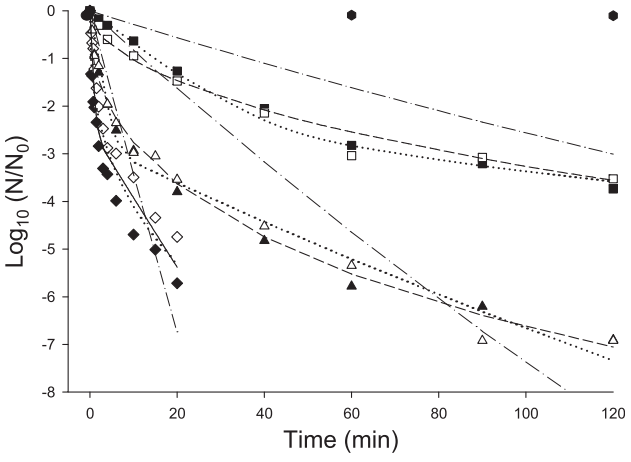

Fig. 7 – Inactivation data of MS2 after exposure to an initial concentration of 0.39 and 0.45 mg/L ClO_2 (diamonds), 0.1 and 0.1 mg/L ClO_2 (triangles), 0.019 and 0.02 mg/L ClO_2 (squares) and 0 (hexagons) mg/L ClO_2. Measured data are presented as data points, and disinfection experiments were performed in duplo (closed symbols, first experiment, open symbols, duplo experiment). For each concentration, the best fit of the MCW model (dash-dot-dash line), the EFH model (dashed line) and the Modified Cerf model (dotted line) to the measured data are plotted.

3.6. *Presence of resistant subpopulations, implications for drinking water disinfection*

Inactivation curves showing tailing during disinfection of viruses have been observed regularly. The EFH model, empirically designed to calculate *Ct* values can describe the inactivation curve accurately, but EFH parameters k, n and m are determined for specific conditions tested. Using these constants for extrapolation of the EFH model outside the tested conditions may lead to a non-accurate calculation of *Ct* values due to complicated inactivation kinetics (Thurston-Enriquez et al., 2003). Furthermore, the empirical basis does not aid in translating model data to biological mechanisms occurring during inactivation. The Cerf model also calculated an accurate inactivation curve, and the mechanistic background aids in understanding inactivation kinetics. The good fit of this model is an indication for the presence of two subpopulations.

Our understanding regarding tailing of viruses during disinfection is limited, but heterogeneity of micro-organism populations as major cause has been discussed (Hiatt, 1964; Najm, 2006). Presence of resistant subpopulations for viruses (as intrinsic population property or acquired after synthesis in the host) may have major implications for the disinfection efficacy of these viruses. For example, it is for viral pathogens not known whether the population in the environment exists of several subpopulations, how much of the population is represented by subpopulations (e.g. 10% or 0.1%) and how much they differ in their resistance against disinfectants. It is also not known whether they may acquire additional resistance in the environment, e.g. by attachment to naturally present particles. Finally it is unknown how resistant subpopulations behave in the environment and whether the disinfectant-resistant virus population may also be able to survive better under environmental conditions. In that case, the more resistant subpopulation will represent a significant part of the infectious virus population in source water.

Viruses representing the tail of the inactivation curve require additional *Ct* credits to be inactivated. By applying *Ct* values derived from the rapidly inactivating population, the ClO_2-resistant subpopulation is neglected and inactivation of this population during disinfection will be lower than expected, posing a hazard for human health. For reliable *Ct* values it is necessary to verify whether the viral inactivation rate is constant over the range that is targeted to inactivate at the disinfectant concentration applied (Hoff, 1986).

4. Conclusions

ClO_2 inactivated bacteriophage MS2 for at least 5 log units after exposure to ClO_2 concentrations from 0.5 mg/L down to 0.02 mg/L. Exposure to 0.005 mg/L ClO_2 resulted in the inactivation of 1 log unit in 300 min.

All MS2 inactivation curves showed significant tailing, and the transfer from fast to slow inactivation occurred after 3 log units of the population had been inactivated. A diminishing biocidal effect during disinfection can be excluded as a reason for tailing. Both the EFH model and the Modified Cerf model,

designed to describe non-linear curves, produced a good fit to the inactivation data.

The observed tailing may be the consequence of intrinsic virus population heterogeneity, or acquired after virus synthesis by attachment to other (virus)particles. Presence of subpopulations is not taken into account when Ct values are being calculated for the inactivation of viruses from surface water sources for drinking water. This could result in passage of the disinfection train for the most resistant viruses.

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