Impact of virus aggregation on disinfection

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Master Thesis

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Abstract:

This study is based on the hypothesis that viral aggregation slows the inactivation by chemical disinfectants. This slow-down depends on the aggregate size, the concentration and the reactivity of the disinfectant. In this work we test this hypothesis by experimentally investigating the inactivation MS2 coliphage aggregates by dichloramine and PhiX174 aggregates by PAA. The experiments performed with MS2 and dichloramine show good adherence to the theory. We observed that inactivation by dichloramine was slowed by 3.4 to 13 times with aggregates of size between 420 and 580 nm, and with a dichloramine concentration varying between 3.5 and 13 mg/L. The larger the aggregates, and the higher the concentration of disinfection, the greater was the observed inhibition of inactivation due to aggregates. Comparing the inactivation of MS2 by dichloramine and by PAA, we observed that dichloramine was a stronger disinfectant, and the impact of aggregation on dichlormaine was more extensive. This permitted to verify the hypothesis that a more reactive disinfectant was more sensitive to aggregation. Our results were furthermore in good agreement with the mechanistic model for the inactivation of viral aggregates developed by Mattle et al. (2011).

In contrast to MS2, results for the disinfection of aggregates of PhiX174 did not exhibit an effect of aggregation on disinfection. We hypothesize that PhiX174 viruses within aggregates were not sufficiently compact to result in inhibition of disinfection. This phenomenon was probably due to the spikes on the PhiX174 capsid. Structural features of viral aggregates thus influence their propensity to inhibit inactivation.

Résumé en français:

Ce travail est basé sur l'hypothèse que les virus en agrégats ralentissent le processus de désinfection chimique. De façon à ce que l'importance de cet impact dépende de la taille des agrégats, de la concentration et de la réactivité du désinfectant. Dans cette étude nous testons l'hypothèse de base en menant des expériences sur l'inactivation du bactériophage MS2 en agrégats par la dichloramine et sur PhiX174 en agrégats par le PAA. Nos résultats avec MS2 et la dichloramine concordent bien avec la théorie. Nous observons que pour des agrégats de tailles allant de 450 à 580nm et des concentrations de dichloramine variant de 3.5 à 13mg/L, on obtient une réduction de l'inactivation allant de 3.4 à 13 fois en comparaison à une inactivation sur des virus dispersés. On observe également que plus les agrégats sont gros et plus la concentration de désinfectant est élevée, plus l'impact sur l'inactivation est important. Comme la dichloramine est un désinfectant plus efficace que le PAA (pour MS2), on vérifie

de se faite, qu'un désinfectant très réactif (comme la dichloramine) sera plus pénalisé par la présence d'agrégats qu'un désinfectant moins réactif (comme le PAA). Le modèle développé dans l'étude de Mattle et al. (2011), que nous avons utilisé ici, concorde relativement bien avec nos résultats.

Cependant, les résultats obtenus avec l'agrégation de PhiX174 ne montrent pas d'effet sur la désinfection du PAA. Nous pensons que les agrégats de PhiX174 ne sont pas assez compacts pour avoir un effet d'agrégation comme avec MS2. Nous en avons déduit que c'est probablement à cause de la forme de PhiX174: ce virus possède en effet de petites pointes sur toute sa capside. La structure des agrégats influence donc les capacités à l'inhibition de l'inactivation.

Acknowledgements

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1 Introduction

Each year about 1.5 millions of children, under the age of five, die due to diarrhoea. These infections are caused by bacteria, parasites and viruses and are transmitted in contaminated food or water[1,2]. Sanitation and access to safe drinking water are the most promising ways to alleviate these problems and can be considered as one of the most important challenges for future generations, especially in developing countries. Research is therefore important to improve our understanding of water treatment and improve drinking water quality.

In infected faeces virus concentrations can be very elevated (1'000'000 viruses/g)[3] and they may be present as aggregates.[4,5,6,7,8,9] Depending on environmental conditions these aggregates may disperse or remain aggregated. However, only few studies investigated the influence of viral aggregation on disinfection[7,10]. Therefore, in this project we want to acquire new knowledge about the impact of viral aggregation on disinfection by chemical oxidants.

1.1 viruses

Viruses are small organisms found everywhere in the environment.[11] They are known to be infectious particles. Viruses are not complex organisms and are composed of only two or three parts: the genetic material (DNA or RNA), a protein coat called "the capsid" and some viruses (not all) have a lipid membrane called "the envelope". Viruses can only grow and reproduce in a living cell and many types are able to harm humans.[12]

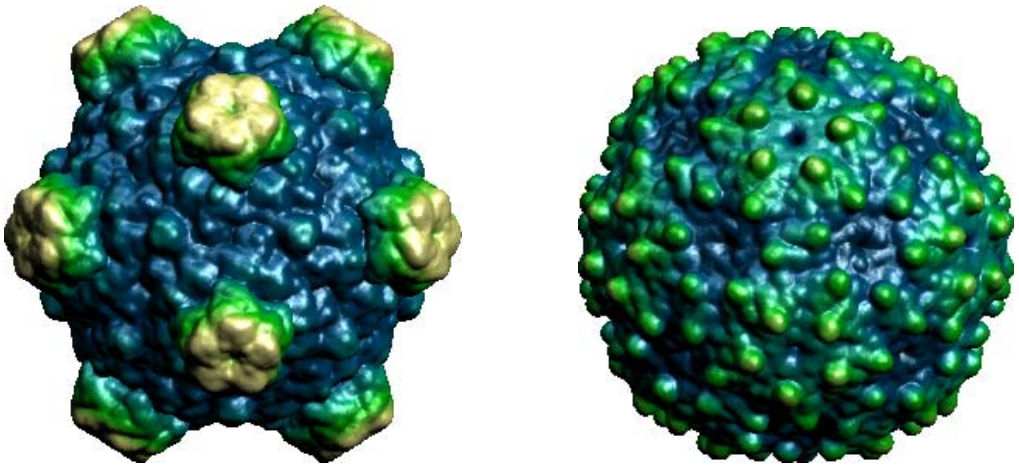

Figure 1:Representation of bacteriophage PhiX174 *(DNA) and bactriophage MS2 (RNA)*[13]

In this study we used bacteriophages as surrogates for human viruses (see Figure 1). The handling of these viruses is much easier as they do not cause any infections to humans. Additionally, results from inactivation studies can be obtained fast as culturing of bacteriophages takes only hours compared to days for certain human viruses.

Moreover, specifically in this study we need to re-disperse the viruses after disinfection treatment. If we work with viruses that aggregate under environmental conditions (around neutral pH), it will be difficult to disperse these aggregates before enumeration. Therefore, we have chosen viruses that aggregate at lower pH values and are often used as surrogates for human viruses: MS2[14] and PhiX174[15]. They have isoelectric points (Ip) of 3.9 and 6.6[16], respectively and a maximum aggregation rate at pH 3 and 5 [14,15].

1.2 Disinfection

More than 2.5 billions people do not have access to improved sanitation and about one billon use unsafe drinking water that causes various diseases[1]. Water and wastewater treatment is an efficient way to break the disease circle. Different treatment technologies like, filtration, disinfection, sedimentation or coagulation, are implemented to improve water quality. However, as viruses are very small (18-120nm) they can pass through filtration processes and may not efficiently coagulate or sediment; therefore disinfection is the treatment of choice to reduce virus concentrations.

A disinfectant can act in two different ways:

- inhibition effect: growth, multiplication is stopped
- lethal effect: death of organisms

As water treatment should reduce microorganism, especially the second kind of effect is required[17]. The modes of action for viral disinfection are not yet entirely understood, however, disinfectants can either affect viral proteins or genome[18].

Among the main properties determining the efficiency of a disinfectant are its concentration, pH, contact time and temperature[8]. Each organisms has its specific resistance to a certain disinfectant but conditions like aggregation seem to have a major impact on the efficiency, too[8,14].

1.3 Disinfectants

A broad range of disinfectants are employed for water treatment, however, in this study we worked under acidic conditions, therefore we have chosen chloramines and chlorine (could only be used under slightly acidic conditions) and peracetic acid (PAA).

1.3.1 Chloramines:

Chloramines, in the form of monochoramine (NH_2Cl), are largely used in USA for secondary disinfection of drinking water. Due to its stability it is practical to maintain a residual disinfectant concentration and prevent re-contamination in the water network. Chloramines are also used in wastewater treatment[19,20].

Besides monochloramine, two additional chloramine species exist: dichloramine ($NHCl_2$) and trichlormaine (NCl_3). However, these two species are generally considered as by-products in water treatment, due to their greater toxicity (especially for trichloramine)[21,22]. The generation of these different products is dependant on pH and on initial free chlorine and ammonia concentrations (see Figures 2 and 3).

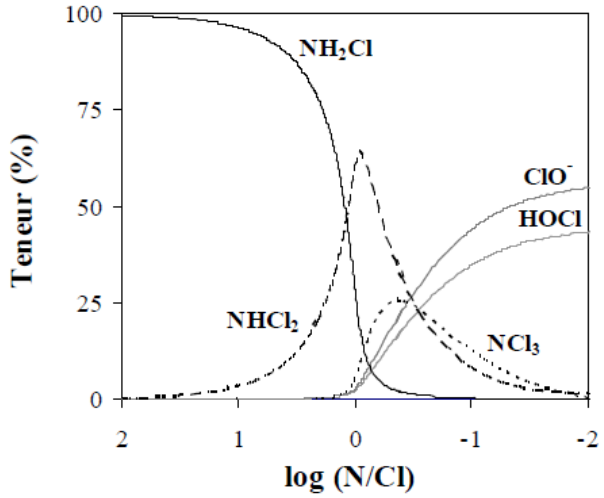

Figure 2: Repartition of chloramines species in function of the ratio N/Cl [21]

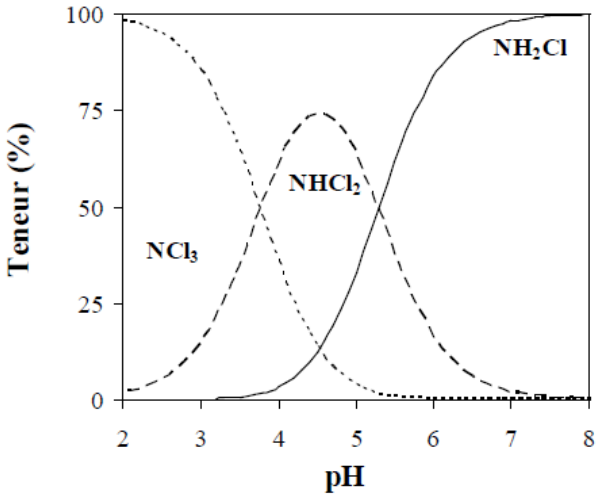

Figure 3: Repartition of Chloramines in function of pH [21]

In this study we worked with dichloramine due to the low pH needed for MS2 aggregation. The advantage of dichloramine is its stability compared to trichloramine or chlorine and its better efficiency than monochloramine for inactivation of viruses. Nevertheless inactivation with dichloramine is pH dependent and stocks cannot be used after 7-10 hours[21,23].

1.3.2 Peracetic Acid (PAA)

PAA (CH_3CO_3H) is very stable and easy to use. It is known as relatively pH independent disinfectant even at low pH[8]. PAA is used in wastewater treatment, and sometimes in industries for water disinfection in cooling processes[8,17,24].

1.3.3 Free Chlorine

Free Chlorine (hypochlorous acid (HOCl) and hypochlorite ion (OCl^-)) is a strong oxidant and is largely used as primary disinfectant for drinking water treatment in many countries. Nevertheless, it is difficult to perform laboratory studies with free chlorine at low pH, as it becomes unstable and more reactive with decreasing pH. Additionally, it reacts strongly with all materials, including buffers and organisms, therefore, it is difficult to maintain a stable concentration especially if it initial concentration is low[8,25].

1.4 Effect of viral aggregation

Aggregation is not a well-studied phenomena, but pH, ionic strength, type of virus and its isoelectric point influence aggregation. If the pH is higher than the isoelectric point (pI) of the virus, it is negatively charged and repulsive forces hinder aggregation. However, if the pH is reduced close to or below the pI, these repulsive forces diminish and aggregation can occur[14]. Other parameters, like high ionic strength or organic matter, can shield/reduce repulsive forces and can also lead to aggregation. Previous studies showed that virus aggregation slowed the inactivation process[4,7,14,26]. This is probably due to removal of the disinfectant inside the aggregates by adsorption or consumption (see Figure 4) [14]

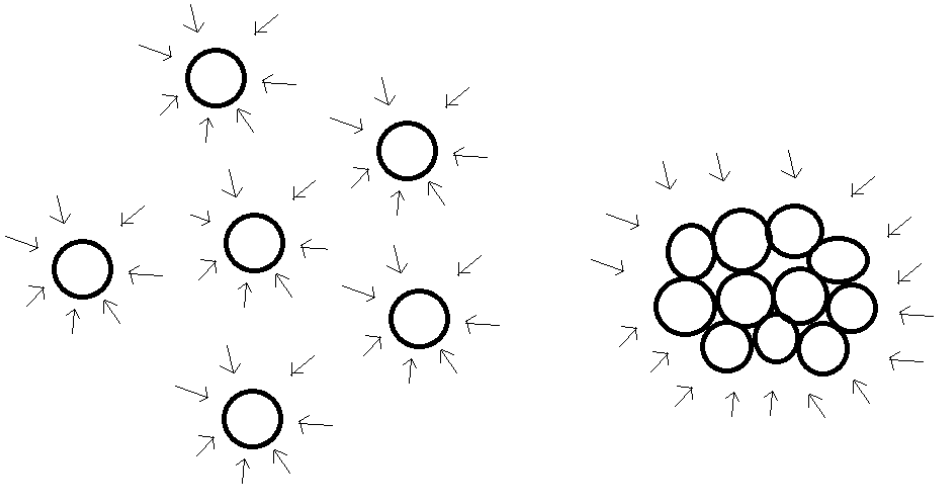

Figure 4: Inactivation on dispersed viruses (left) and aggregated viruses (right)

In water disinfection, the disinfectant concentration is very important and must be chosen correctly for sufficient disinfection and minimum by-product formation. In the case of viral aggregation the disinfectant dose may be underestimated and may jeopardize achievement of the treatment goal.

Further, a study by Mattle et al. showed that the impact of viral aggregation was more important for high disinfectant concentration and for larger aggregates[14]. (see Figure 5)

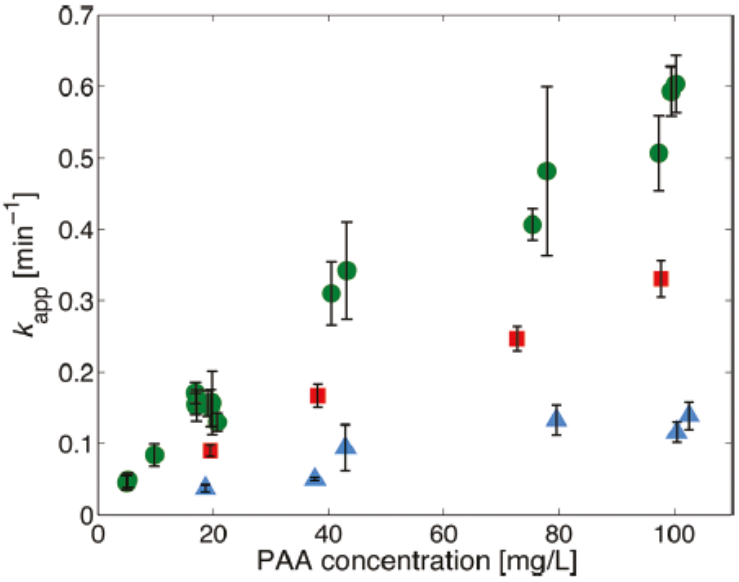

Figure 5: Inactivation rate constants (k_{obs}) of MS2 viruses ($5*10^{10}$ PFU/mL) by PAA: in blue: large aggregates at pH 3, in red: small aggregates pH 3.6, and in green: disperse viruses at pH 5. At high concentrations of PAA the increase of k_{obs} deviates from linearity when aggregates are present. [14]

Additionally, a model was derived that explained the experimental data observed in Figure 5. The model is based on two laws: the first describes the inactivation kinetics:

$$\partial_t c_v = -k_{app} c_v = -k_1 c_d^{\alpha} c_v \qquad (1)$$

where c_v is the concentration of infective viruses, c_d the concentration of disinfectant, k_{app} the apparent inactivation rate constant and k_1 the α^{th} order inactivation rate constant, which is pH dependant.

The second is the reaction diffusion equation:

$$\partial_t c_d = D\nabla^2 c_d - k_2 c_d \qquad (2)$$

where k_2 is the rate constant associated with removal of the disinfectant due to reaction and D is the molecular diffusion coefficient. To have appropriate boundary conditions we define c_v^0 the concentration of viruses at time 0 and c_d^0 concentration of disinfectant at time 0. We have the hypothesis that c_d is constant over the experiment, which implies the simplification $c_d(R,t) = c_d^0$.

At equilibrium, equations (1) and (2) with boundary conditions become:

$$c_v(r,t) = e^{-k_1\left(c_d^0 \frac{(r\sqrt{k_2/d})^{-1/2} I_{1/2}(r\sqrt{k_2/d})}{(R\sqrt{k_2/d})^{-1/2} I_{1/2}(R\sqrt{k_2/d})}\right)^{\alpha} t} \qquad (3)$$

Thus we can compute the average inactivation of viruses within the aggregate numerically by integrating

$$\bar{c}_v(t) = \frac{3}{R^3}\int_0^R r^2 c_v(r,t)dr \qquad (4)$$

where r is the distance of diffusion inside the aggregate, R the radius of the aggregate and t the time.

This model allows us to calculate the average virus concentration depending on the size of aggregate R and the concentration of disinfectant c_d^0.The model furthermore predicts that aggregates should have a greater effect on reactive disinfectants, whereas mild disinfectants maintain their efficiency even in the presence of large aggregates[14]. However, previously this model was only tested with one disinfectant (PAA) and for a single virus (MS2).

1.5 Goals of the study

The goal of this study wants to evaluate whether the model predictions put forward in the work by Mattle et al[14]. are applicable for other disinfectants and viruses. In particular, two objectives were pursued:

1) Establish and compare the effect of aggregation on inactivation kinetics of a single virus (MS2) by different disinfectants

2) Determine and compare the effect of aggregation on inactivation of different viruses by a single disinfectant (PAA)

We used bacteriophages MS2 and phiX174 as surrogates for human enteric viruses. PAA, chloramines (dichloramine) and chlorine for were chosen as disinfectants. Disinfection was studied as a function of:

- Aggregate size
- Disinfectant concentration
- Disinfectant reactivity

2 Materials and methods:

We performed three different kinds of experiments: MS2 was inactivated by dichloramine, PhiX174 by PAA and by free chlorine. In short, aggregated and dispersed samples of viruses were inactivated by a disinfectant, inactivation rate constants were determined and modelled. The viruses were first aggregated by lowering the pH, the disinfectant was than added, and finally, before enumeration of the remaining infective viruses, the aggregates were re-dispersed by increasing the pH. Different pH values for the different bacteriophages were chosen to conduct the inactivation experiments under aggregating (pH 3 for MS2 and 5 for PhiX174) and dispersing conditions (pH 4 for MS2 and 6 for PhiX174). Variations in aggregate sizes were obtained by increasing or decreasing virus concentrations. Finally, to evaluate the pH effect on inactivation, control experiments in high phosphate concentrations (400 mM for MS2 and 200 mM for PhiX174) were performed, as under these conditions no aggregation occurred.

2.1 Buffers, Organisms and culturing methods:

All buffers, Organisms and culturing methods used are described in appendix.

2.2 Disinfectants

Three different disinfectants were used. To avoid disinfectant degradation, protocols of production were established and strictly followed for all experiments. A short description of the experimental details is given here, additionally, detailed protocols can be found in the appendix.

2.2.1 Chloramines:

Chloramines were produced by mixing ammonium chloride (NH_4Cl) with sodium hypochlorite (NaOCl)[22]. A ratio of Cl_2 [mg/L]/NH_4 [mg/L] = 4[21] and a concentration of 2.2 - 4.4mM/L of Cl_2 permitted the production of the wanted chloramines avoiding the generation of trichloramine. Dark bottles were used to avoid light degradation. The first reaction at pH 8.4 created monochloramine (NH_2Cl) very quickly. 20 minutes later, the pH was lowered to 4 by the addition of hydrochloric acid to initiate the transformation of monochloramine to dichloramine. To achieve complete conversion, the solution was kept at the same pH for 2

hours. During the transformation process the pH had to be constantly re-adjusted (with HCl) during the first hour. Dichloramine was used within 4 hours to avoid trichloramine formation[21].

Two methods were used to determine chloramines concentrations. The initial chloramine concentration was determined by a direct absorbance measure at 254 nm (monochloramine peak) and 294 nm (dichloramine peak)[21] However, as the virus absorbance was in the same range, this method could not be used once the experiment was initiated. Therefore, the DPD colorimetric method[27] at 515nm using a spectrophotometer (UV-2550 Shimadzu) was used to control disinfectant stability during the experiments. This method determines the combined residual chlorine (CRC) and consists of two separate measures: the first for free residual chlorine (FRC) and the second for the total residual chlorine (TRC). The chloramine concentration was calculated by subtracting FRC from TRC: CRC = TRC – FRC[27]. See the appendix for protocols and calculations.

2.2.2 Peracetic acid (PAA)

The PAA stock solution was prepared each day by diluting PAA into mili-Q water to obtain a 5 g/L PAA solution. PAA concentrations were determined by the KI colorimetric method[28] (see appendix) at 352 nm.

2.2.3 Free chlorine (HOCl)

The HOCl stock solution was prepared each day by diluting sodium hypochlorite (NaOCl) into mili-Q water to obtain a 1.5 g/L HOCl solution. Due to the high reactivity of free chlorine, all materials (tips, plastic cuvettes, plastic tubes and glass flasks) were pre-treated in a chlorine bath for 12 hours before use.

Free chlorine concentrations were determined by the DPD colorimetric method[27].

2.3 Size measurement with dynamic light scattering (DLS)

We have performed the experiments with Zetasizer Nano ZS (Malvern instruments). The measurements were run at high resolution in backscattering mode (173°) with temperature fixed at 22°C. Each measurement yields the z-average value in radius and takes about 3.8 minutes and contains 21 runs of 11 seconds each.

2.4 Viral aggregation and dispersal

Aggregation was induced by lowering the pH below the isoelectric point (pI). This reduced repulsive forces due the negative charges of viruses at neutral pH[14]. An increase of the pH above the pI redispersed the viruses and allowed enumeration of single viral particles that were inactivated within aggregates.

2.4.1 MS2

The same virus stock solution ($5*10^{12}$ pfu/mL) was used for all experiments which were performed in 15 mM phosphate and 15 mM chloride and at pH values of 3 and 4 for aggregated and dispersed experiments, respectively (pI of MS2 = 3.9[16]). The viruses were left to aggregate (at $5*10^{10}$ pfu/mL for 450 nm radius and at $1*10^{11}$ pfu/mL for 580 nm radius) for at least one hour before the disinfectant was added, and the growth of the aggregate size was recorded in real-time by the DLS for each experiment. An increase of initial virus concentration permitted to increase the aggregate size under the same experimental conditions. After the inactivation with the disinfectant, the remaining viruses were re-dispersed by diluting them into phosphate buffer saline (PBS, 5mM phosphate 10mM chloride at pH 7.5) which increased the pH above 7.0.

In order to avoid aggregation and determine the pH effect, control experiments were conducted in high phosphate buffer solutions (400 mM phosphate and 15 mM chloride). The pH was increased above 7.0 after inactivation by high phosphate PBS (150 mM phosphate, 10 mM chloride and pH 7.5).

2.4.2 PhiX174

The experiments conducted with PhiX174 were basically the same as for MS2, however, experiments performed under aggregating and dispersing conditions were performed at pH 5.0 and 6.0, respectively. The pI of PhiX174 was reported to be at 6.6[16] but we only observed[8] aggregation close to pH 5.0. Additionally, the high phosphate buffer contained only 200 mM phosphate instead of 400 mM for MS2, as strong aggregation was recorded at 400 mM phosphate for PhiX174.

The dispersal was performed using the same buffer solutions as for MS2.

2.5 Inactivation

The disinfection experiments were conducted inside the DLS to maintain the temperature at 22°C and follow the evolution of aggregation size in real-time. All experiments were performed in 15mM phosphate and 15mM chloride, except for experiments in high phosphate buffers (400 mM phosphate and 15mM chloride). Inactivation versus time was modelled by a first-order fit, resulting in inactivation rate constants k_{app}.

2.5.1 MS2 inactivation by Dichloramine

Once complete dichloramine transformation was achieved, 8 mL phosphate buffer were mixed with 2 mL dichloramine stock solution and mili-Q water to achieve the desired final disinfectant concentration. The pH of the solution was re-adjusted (with HCl and NaOH). Another measurement with the spectrophotometer was performed (with both DPD and direct absorbance methods) in order to exactly determine the initial dichloramine concentration.

A 1 mL cuvette containing 0.5 mL phosphate buffer without disinfectant was introduced into the DLS. Then 10 µL of MS2 stock solution were added (virus concentration at this step are: $1*10^{11}$ pfu/mL or $2*10^{11}$pfu/mL to obtain bigger aggregate). Size measurements were recorded over the whole experiment every 3.8 minutes. The viruses were left to aggregate for 1 hour (this step was omitted for pH 4.0 and for high phosphate experiments). Buffer containing dichloramine was then added (0.5 mL) and gently mixed. The dichloramine concentrations varied between 7 and 26 mg/L for the different experiments with an average loss over experimental time less than 5%. Samples were collected periodically: 10 µL for the first sample before the addition of disinfectant (t_0), and 20 µL for all the remaining ones. These samples were diluted in 240 µL PBS (high or low phosphate depending on the experiment) to raise the pH and redisperse MS2, and were amended with 240 µL sodium thiosulfate solution (350 mg/L) to quench the remaining dichloramine. Finally, diluted samples were plated and results counted 12 hours later.

2.5.2 PhiX174 inactivation by PAA

PAA solutions contain hydrogen peroxide, which is known to generate hydroxyl radicals in the presence of trace amounts of metal ions[29]. To avoid virus inactivation by hydroxyl radicals, all the phosphate buffers were spiked with EDTA (total concentration of 2 µM/L and 20 µM/L EDTA for low and high phosphate buffers, respectively) to complex the trace metals and prevent radical formation. PAA stock solution (5g/L) was added to the phosphate buffer

to achieve the wanted final concentrations (the pH was re-adjusted with HCl and NaOH for obtaining the initial value of the buffer), which was confirmed with the spectrophotometer (KI colorimetric method[28]).

0.25 mL of phosphate buffer (without EDTA) were placed in a 1 mL cuvette and put in DLS. Then 5µL PhiX174 viruses ($2*10^{12}$ pfu/mL) were added and mixed. As for MS2 experiments, the samples were left to aggregate for 1 hour (excepted for pH 6 and for high phosphate). Next, 0.75 mL of buffer with PAA was added and gently mixed to initiate the inactivation step. The PAA concentrations varied between 16 and 23 mg/L for the different experiments with an average loss during the experiment of less than 0.1%. The additional steps were the same as for the MS2 experiments, however, the volumes changed: 5µL for t_0 and 40 µL for the other samples were diluted in 200 µL PBS and sodium thiosulfate (350 mg/L). During the experiment two other PAA measurements were performed using KI colorimetric method to determine the PAA stability.

2.5.3 PhiX174 inactivation by free chlorine

The free chlorine stock (14%) was diluted in phosphate buffers to obtain the wanted concentration (in a treated tube from the chlorine bath), then the pH was re-adjusted, and the sample was stabilized for 10 minutes to achieve a constant free chlorine concentration. The concentration was determined with DPD colorimetric method[27].

We placed 0.9 mL phosphate buffer solution in untreated 1 mL cuvette and added 9 µL PhiX174 ($2*10^{12}$ pfu/mL) solution. In the DLS, the solution at $2*10^{10}$ pfu/mL was left to aggregate for 1 hour (except for pH 6 experiment).

To a 4 mL cuvette and with tips both treated in the chlorine bath, 4 mL buffer solution containing free chlorine was added. The initial concentration was determined immediately before the addition of the viruses to the solution (DPD colorimetric method). Then, the 4 mL cuvette was inserted into the DLS and 300 µL of the previously prepared virus solution was added. Samples of 200µL were periodically withdrawn and diluted in 200µL PBS and 200µL sodium thiosulfate solution (350 mg/L). An additional concentration measurement (colorimetric DPD method) was conducted at the end of the experiment (we have loss about 40%). Samples were enumerated as described above.

3 Results and Discussion

3.1 Aggregation

At neutral pH, most viruses are negatively charged and therefore repulse each other, resulting in a dispersed state. Aggregation can, however, be induced by lowering the pH to values close or below the pI of the corresponding virus. Other factors (ionic strength, organic matter content) can also influence aggregation. It was found in a previous study[8] that high ionic strength resulted in dispersing conditions even at pH values close to the pI. This phenomenon could be explained by the shielding of the negatively charged genome within the viral capsid which results in a positive charge observed at the outside of the virus.

3.1.1 Aggregation of MS2

Aggregation of MS2 is well documented in the Mattle et al. (2011) study. We have chosen to perform experiments at pH 3 to achieve more efficient aggregation (see Figures 6 and 7).

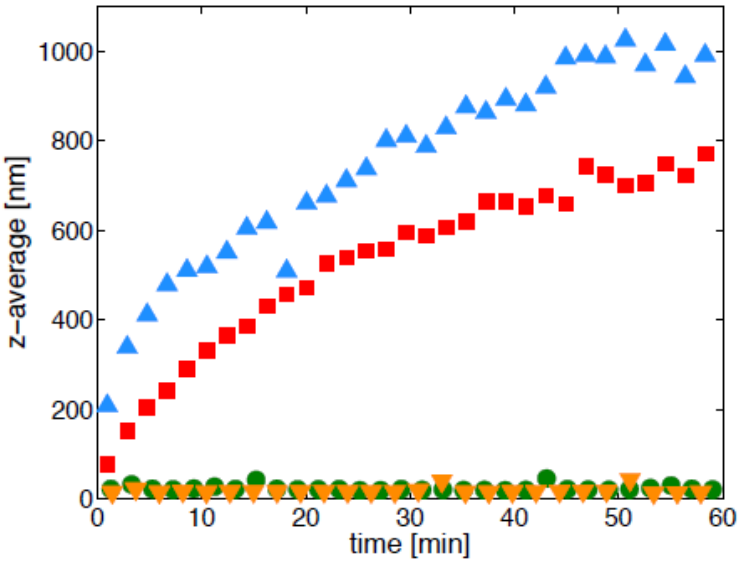

Figure 6: Figure from Mattle et al (2011) show the evolution of MS2 aggregates size in 60 minutes. In blue: experiment at pH 3 15mM of phosphate; red: pH 3.6 15 mM of phosphate; orange and green: pH 3 and 3.6 with 400 mM of phosphate. Concentrations of viruses were of $5*10^{10}$ pfu/mL.

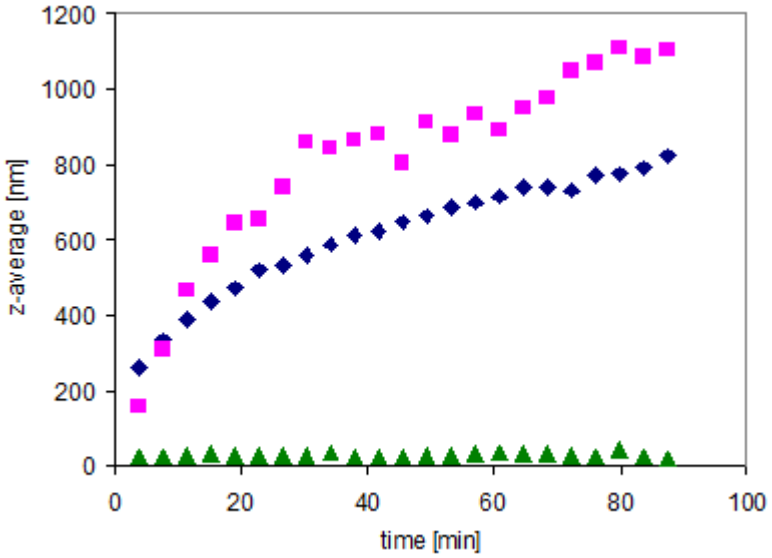

Figure 7: Figure from present work, showing the evolution of MS2 aggregates size in 90 minutes. In pink: pH 3, 15mM phosphate, $1*10^{11}$pfu/mL; in blue: pH 3, 15mM phosphate, $5*10^{10}$pfu/mL; and in green: pH 4, 15mM phosphate, $5*10^{10}$pfu/mL.

Aggregation formation was similar in this study under the same conditions (pH and ionic strength). As can be seen in Figure 7, doubling the MS2 concentration (pink squares) led to an increase in aggregate size compared to the lower virus concentration under the same solution conditions (blue diamonds). After one hour, the aggregate radius corresponded to approximately 700-800 nm for the lower virus concentration, and 900-1000 nm for the higher concentration.

Due to the addition of disinfectant, the average aggregate size temporarily reduced (decrease from 800nm to 350-400 nm), and later re-grew over the experimental period (see Figures 8 and 9). The dispersion after the addition of dichloramine looked the same as the dispersion due to PAA and the re-growth rate was equivalent. Therefore, we can conclude that the dispersion effect was only due to the mixing and the slower re-growth to the dilution (by a factor 2) after disinfectant addition.

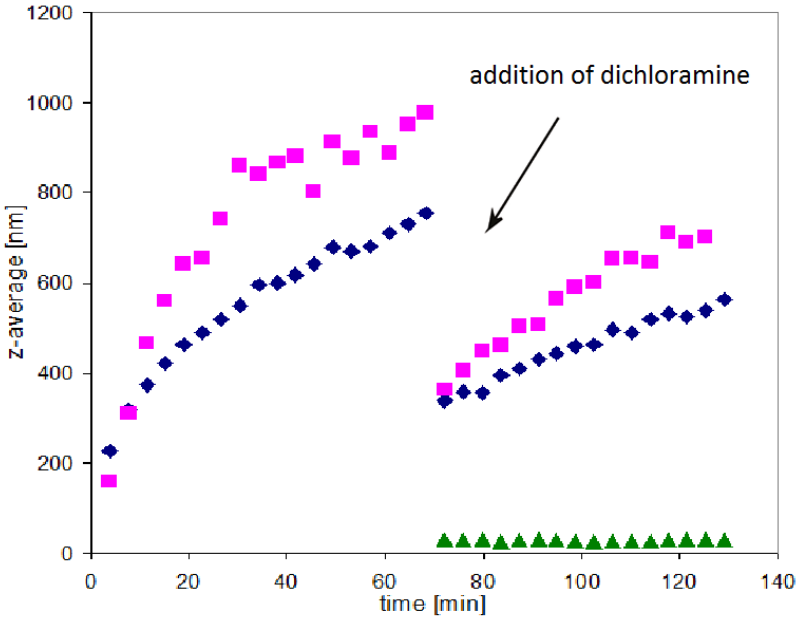

*Figure 8: MS2 aggregate formation at pH 3.0, 15mM phosphate, 5*10^{10} pfu/mL (blue) and 1*10^{11}pfu/mL (pink) and at pH 4.0, 15mM phosphate, 5*10^{10} pfu/mL (green) . Dichloramine was added after one hour of aggregation. leading to a decrase in aggregate size.*

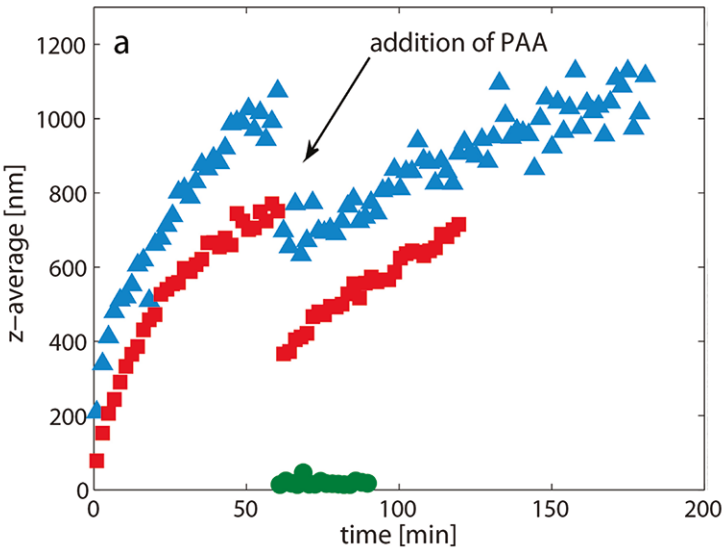

*Figure 9: From Mattle et al (2011); MS2 aggregate formation (at pH 3.0 (blue), pH 3.6 (red) and pH 5 (green), 15mM phosphate, 5*10^{10}pfu/mL). PAA was added after one hour of aggregation.*

3.1.2 Aggregation of Phi X-174

The optimal aggregation efficiency was determined by several aggregation experiments in the pH range from 4.3 to 6. The fastest aggregation was found at pH 5.0 and no aggregation was observed at pH 6.0 (see Figure 10).

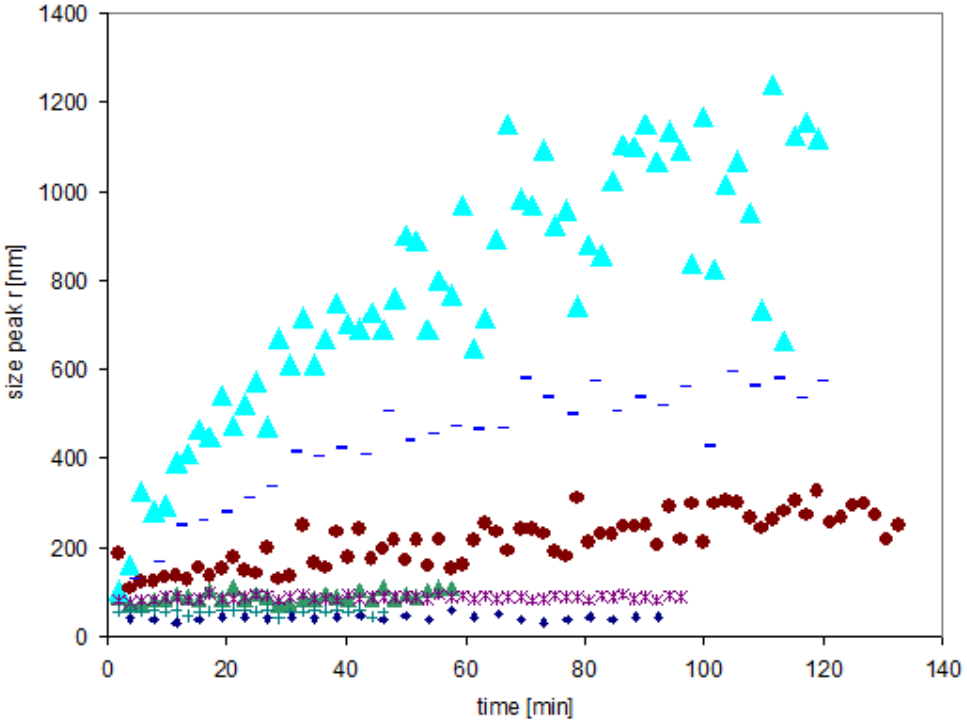

Figure 10: aggregate formation (light blue triangles: pH 5, 15mM phosphate, $1.2*10^{11}$pfu/mL) and (15mM phosphate, $2*10^{10}$pfu/mL, pH 5 blue rectangles, pH 4.8 brown rounds, pH 4.5 purple stars, pH 5.2 green triangles, pH 6 blue lozenges, pH 6.6 green crosses).

In contrast to MS2, PhiX174 aggregated in at a of pH values of about 5 instead of pH 3. Additionally, aggregates were more easily re-dispersed due to mixing.

In order to remain under dispersing conditions at pH 5.0, only 200 mM of phosphate were necessary. In 400 mM phosphate buffer, however, aggregation occurred at even at a faster rate than in low phosphate. Aggregation is a complicated phenomenon and for now we do not yet understand the loss of repulsive forces under this high phosphate concentration (see figure 11).

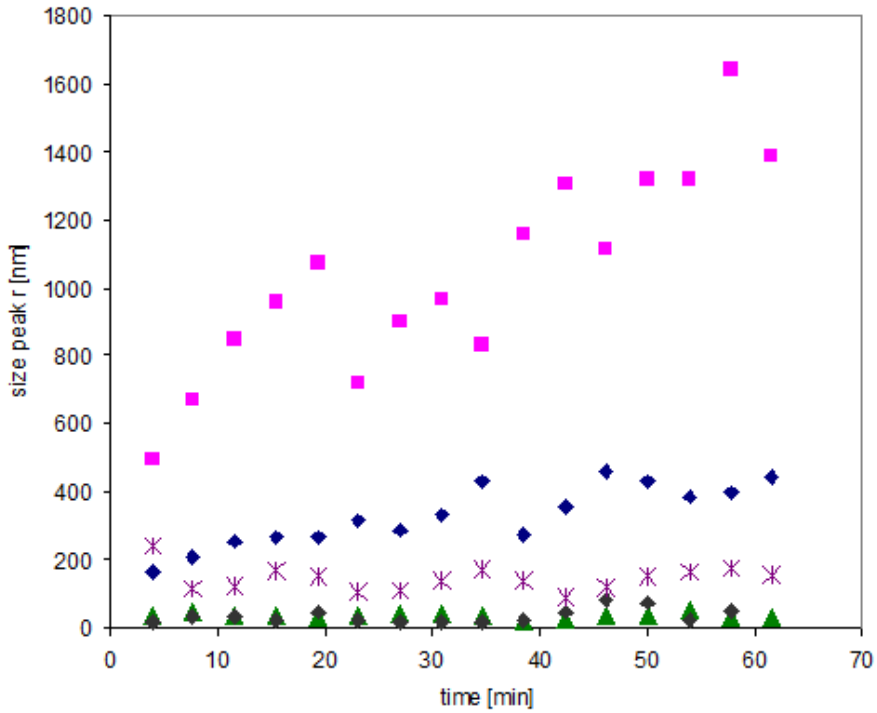

Figure 11: Aggregation check at pH 5.0, 15mM (blue) / 150mM (grey) / 200mM (green) / 250mM (purple) / 400mM (pink) phosphate and 15mM chloride

3.1.3 Aggregation of FR

Aggregation experiments were also performed with FR. This virus was chosen because of its reported pI (9.0)[16], which is surprisingly elevated. However, aggregation looked very much the same as for MS2 and no aggregation was observed at elevated pH values. Due to the apparent similarity between MS2 and FR (aggregation and structure), no disinfection experiments were conducted with FR.

3.2 MS2 inactivation by dichloramine

In the literature, little information exists about dichloramine inactivation of viruses at low pH. In general, inactivation studies were performed at neutral pH and with monochloramine. In our study the use of dichloramine was necessary due to the low pH values under which monochloramine converts into dichloramine. The choice to perform dispersed experiments at pH 4.0 and not 5.0, as used previously with PAA, was to avoid interference from monochloramine formation at higher pH values.

3.2.1 Effect of pH and phosphate concentration on inactivation

Inactivation kinetics with chloramines are strongly pH dependant, because at different pH values different chloramines species are present: mono/di/tri/chloramines. All species have different inactivation kinetics. However, literature on the subject is scarce and contradictory: some studies found that monochloramine was the better disinfectant for bacteria and very inefficient for viruses[23,30] and others revealed exactly the opposite[31,32,33,30]. Preliminary inactivation experiments at solution pH favouring monochloramine showed that monochloramine was a weaker disinfectant compared to dichloramine for MS2 inactivation (data not shown).

The pH values chosen in our experimental system (3 and 4) should favour the presence of dichloramine. Nevertheless, pH may influence dichloramine disinfection kinetics under our experimental conditions. To determine the effect, experiments were conducted at high phosphate concentration, where viruses are in a dispersed state at pH 3 as well as 4. This allowed us to determine the effect of pH without confounding effects of aggregation.

The experiments performed under dispersing conditions (400 mM phosphate) showed an important pH effect for dichloramine between pH 4.0 and 3.0 (See figure 12)

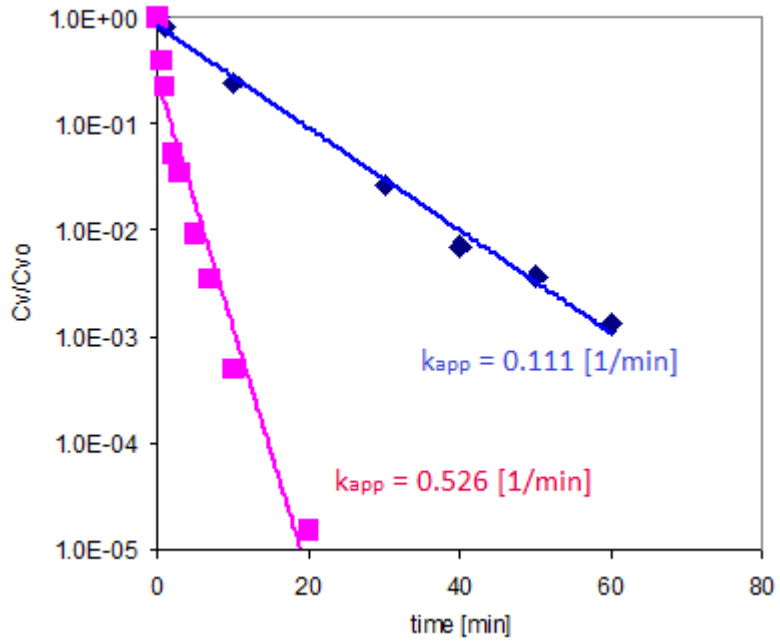

Figure 12: MS2 inactivation at high phosphate concentrations (400mM) by dichloramine (7mg/L) at pH 3 (pink) and pH 4 (blue), virus concentration of $5*10^{10}$pfu/mL

The pH effect was determined from the ratio of the two k_{app}:

$$\frac{k_{app}(pH3\_400mM)}{k_{app}(pH4\_400mM)} = \frac{0.526\,\mathrm{min}^{-1}}{0.111\,\mathrm{min}^{-1}} = pH\_effect = 4.74$$

Even though this factor was determined under high phosphate concentrations, we assume that the pH effect is phosphate-independent. Therefore this factor was applied as a correction factor to low phosphate experiments (at pH 4) to compare experiments conducted at these different pH values.

Additionally, experiments performed at high phosphate concentration showed lower k_{app} values compared to low phosphate concentrations (see figure 13).

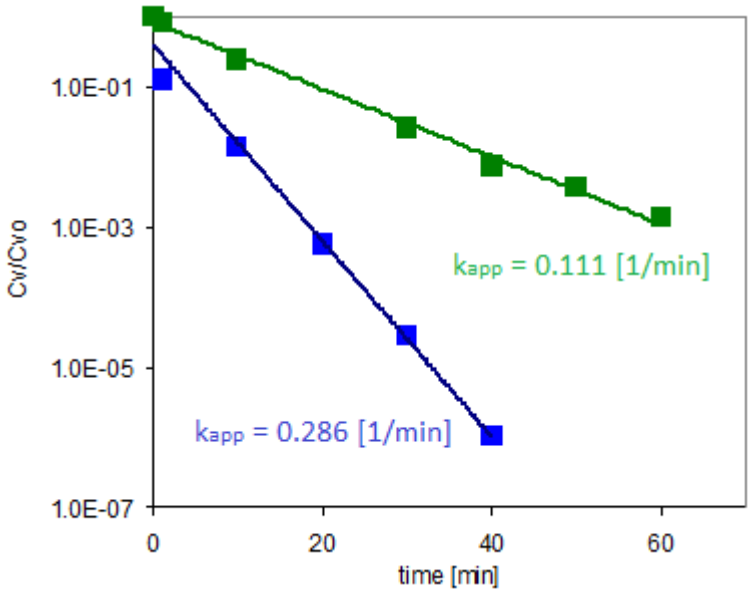

Figure 13: MS2 inactivation at high phosphate concentrations (400mM) in green and at low phosphate (15mM) in blue by dichloramine (7mg/L) at pH 4, virus concentration of $5*10^{10}$pfu/mL

The increased phosphate concentrations reduced the k_{app} by a factor of:

$$\frac{k_{app}(pH4\_15mM)}{k_{app}(pH4\_400mM)} = \frac{0.286\,\mathrm{min}^{-1}}{0.111\,\mathrm{min}^{-1}} = phosphate\_effect = 2.58$$

High chloride concentrations were also tested to avoid aggregation at low pH. However, at 15mM phosphate and 400 mM NaCl the inactivation rate constant was highly increased k_{app}=6.8[min^{-1}], therefore phosphate was chosen to keep viruses dispersed at low pH.

3.2.2 Effect of aggregation and dichloramine concentration on inactivation

The aggregation effect was determined for different aggregate sizes (Figure 14) and different concentrations of dichloramine (Figure 15). Note that the data at pH 4.0 were correct for the pH effect: kapp value for pH 4 was multiplied by the pH effect (4.74) .

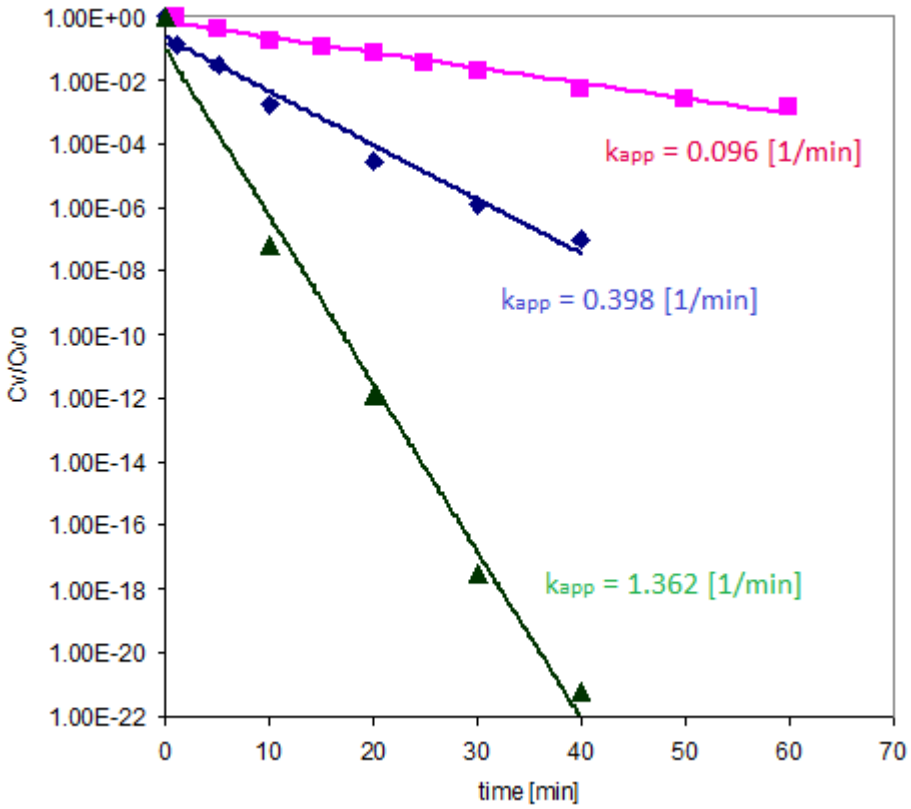

Figure 14: inactivation of MS2 by dichloramine (7mg/L) in pink ($1*10^{11}$pfu/mL, 15mM phosphate, pH 3, aggregates size r 580nm), in blue ($5*10^{10}$pfu/mL, 15mM phosphate, pH 3, aggregates size r 440nm), in green ($5*10^{10}$pfu/mL, 15mM phosphate, pH 4) adjusted to the pH effect.

As for disinfection by PAA[14], inactivation followed pseudo-first-order inactivation kinetics. Despite the presence of aggregates, the data in Figure 14 clearly shows that there are no tailing effects during the disinfection process.

With the data on the different aggregates radius and the pH effect (figure 14) we could estimate the impact of aggregation on MS2 inactivation by dichloramine. To do so, we compared the inactivation kinetic of the different aggregated and dispersed viruses. To obtain a comparison between dispersed and aggregated viruses we need to correct the pH effect. This correction gave us a k_{app} for dispersed viruses of 1.362 min^{-1} instead of 0.286 min^{-1}.

We observed a distinct decrease of the inactivation rate constant with the increase of aggregates size. At the pH 3 and 7 mg/L of dichloramine, the slowest inactivation with a k_{app} = 0.096 min^{-1} was observed for experiments with biggest aggregates (580 nm of radius). When the aggregates were smaller (440 nm of radius) the disinfection process was also slowed but to a less important extent (k_{app} = 0.398 min^{-1}). Finally at pH 4 where no aggregates were obtained (and with the correction of pH effect), inactivation was fastest with a k_{app} = 1.362 min^{-1},

Thus far we only showed the aggregation effect for one concentration (7 mg/L of dichloramine). However we observed changes of this effect with varying disinfectant concentration. As described in equation (1), k_{app} varies with the disinfectant concentration according to: $k_{app} = k_1 c_d^{\alpha}$

In spite of the small number of data in dispersed conditions, we can deduce a saturation effect at high disinfectant concentration as previously observed for other disinfectants[14,34,35].
We expressed the k_{app} values with the concentration in the figure 15

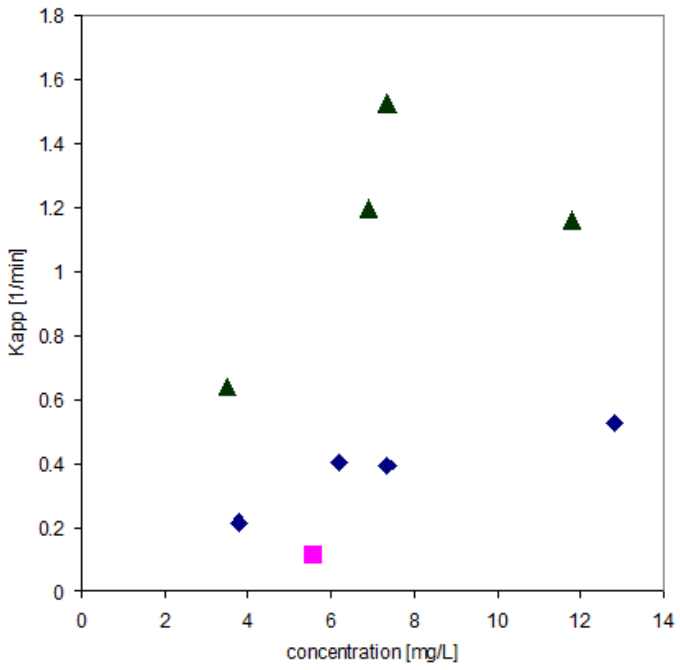

Figure 15: k_{app} versus dichloramine conc. (at pH 3.0, 15mM phosphate, aggregate radius 450nm, $5*10^{10}$pfu/mL in blue), (at pH 3.0, 15mM phosphate, aggregate radius 580nm, $1*10^{11}$pfu/mL in pink) and (at pH 4.0 corrected of pH effect, 15mM phosphate, $5*10^{10}$pfu/mL in green).

We can observe on this figure that the k_{app} values for dispersed viruses were higher than k_{app} values of aggregates for all dichloramine concentrations tested. Moreover, the saturation effect seems more important for aggregates dispersed viruses. To obtain a better confirmation of this effect more data will be needed. However, given that the trend of our results is in agreement with the study of Mattle et al. (2011), we can suggest that with the aggregation effect is more pronounced for elevated disinfectant concentrations.

To conclude, aggregation has a very important effect for MS2 inactivation by dichloramine. In comparison with the results reported for MS2 inactivation by PAA[14], we found that aggregation decreases k_{app} values for dichloramine more readily and for smaller aggregates. Additionally, dichloramine can be considered a stronger disinfectant than PAA (inactivation was faster than for PAA at the same concentration). *This confirms the hypothesis that stronger disinfectants are more impacted by aggregation than weaker ones*.

3.2.3 Mechanistic Model

The model accounts for two mechanisms: the inactivation kinetics depending on the local concentration of dichloramine and the diffusion of disinfectant into the aggregates. To simplify the equations of the model we make the following assumptions:

- The concentration of dichloramine outside and at the boundary of aggregates remain constant. This hypothesis is justified while the molecular diffusion is able to compensate the absorption or consumption of disinfectant by aggregates.

-Aggregates are considered as spheres with radius R.

-The time scale for the penetration of disinfectant into the aggregates is not relevant for the inactivation process. The diffusion coefficient of dichloramine in water can be estimated[36] as $D=1.14*10^{-9}m^2/s$. This gives us the estimation that only 0.00114s are needed to reach the centre of aggregates (radius 1000 nm). That is why we can only consider the equilibrium variation of concentration inside the aggregates according to the radial coordinate.

Within our modelling framework, the live virus concentration is obtained solving equations (1) and (2) described in the introduction. Since we only have access to averages we further need to compute:

$$\bar{c}_v(t) = \frac{3}{R^3}\int_0^R r^2 c_v(r,t)dr \qquad \text{with} \qquad c_v(r,t) = e^{-k_1\left(c_d^0\sqrt{R/r}\frac{I_{1/2}(r\sqrt{k_2/D})}{I_{1/2}(R\sqrt{k_2/D})}\right)^{\alpha} t}$$

The parameter k_1 was estimated assuming the following law of disinfection for dispersed viruses: $k_{app} = k_1 c_d^{\alpha}$ with the k_{app} (pH 4 low phosphate) adjusted accounting for the effect of pH. From our experimental data, we found a $k_1 = 0.236$ $min^{-1}(mg/L)^{-\alpha}$. The parameter α could normally be found from the fit of k_{app} versus C_d for dispersed viruses (Figure 15). However with only four data points we were not able to sufficiently constrain the fit. The parameters α, along with the parameter $\sqrt{k_2 / D}$, were therefore obtained from fitting the model to the inactivation for aggregated samples. As a result we obtained $\alpha = 0.7$ and $\sqrt{k_2\_best / D} = 9\mu m$ Those values were obtained minimizing the (root mean square) RMS error between the predicted and the experimental values for k_{app}. Table 1 gives an overview over the experimental and fitted data.

Table 1: k_{app} obtain experimentally, and modeled using the corresponding parameters C_d mg/L and R nm

	dichloramine Cd [mg/L]	aggregates radius R [nm]	Kapp_exp [1/min]	Kapp_mod [1/min]
pH 3 low phospahte	7.35	425.5	0.39087	0.37238
	6.19	455	0.40461	0.30176
	3.78	421	0.21553	0.25904
	12.80	410	0.52652	0.55231
	5.59	587	0.10977	0.17675

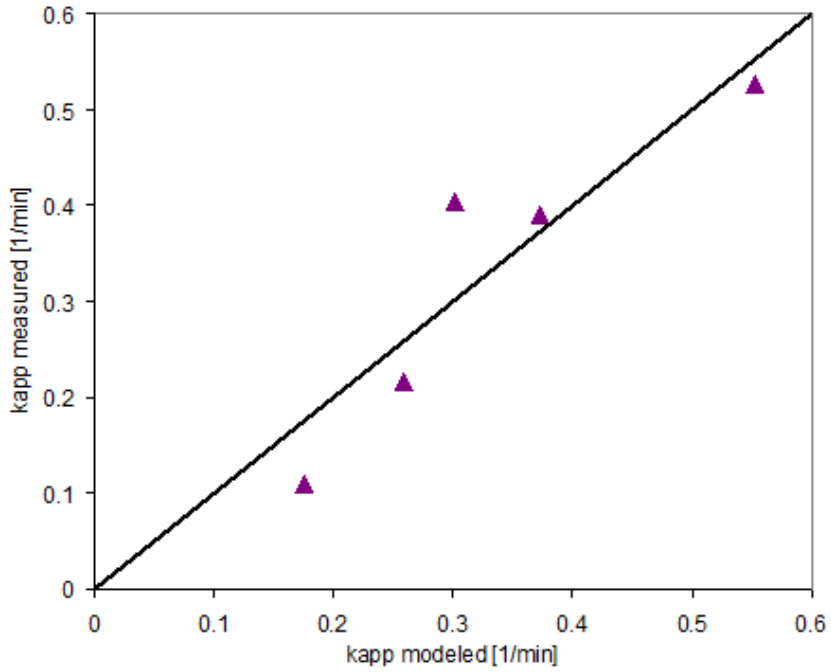

Figure 16: Comparison of experimental and modelled k_{app}. The solid line indicates the 1:1 relationship. Agreement $R^2 = 0.83$

The correspondence between the experiment and the model is $R^2 = 0.83$ (Figure 16). With the amount of data at disposition the fit remains clearly underconstrained.

Based on the fitted parameters, estimates of k_{app} were computed for different aggregate radii. These estimates were compared to those for MS2 disinfection by PAA[14] (Figure 17).

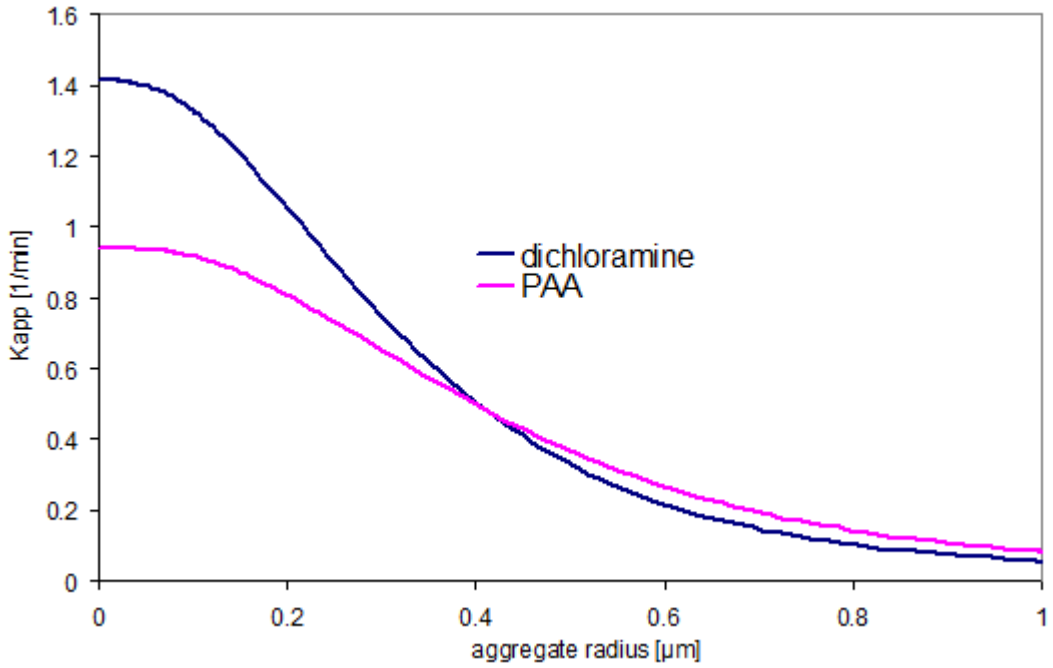

Figure 17: Variation of k_{app} with the aggregate radius. In blue: inactivation by dichloramine [11.1 mg/L]; in pink: inactivation by PAA [100 mg/L][14]

In this figure we can see that dichloramine (at 11.1 mg/L) is more effective for dispersed viruses than PAA (at 100 mg/L). For aggregates of about 0.3-0.4 µm, however, the inactivation by dichloramine becomes less efficient than for PAA. Overall, we observe a very large difference in dichloramine inactivation between the dispersed and aggregated solutions. We further clearly see that for dichloramine, the effect of aggregation depends more strongly on the size of the aggregates than for PAA. This is due to the rapid consumption or removal of dichloramine within the aggregate, as shown in Figure 18. *The modelling results thus confirm that a strong disinfectant such as dichloramine is very efficient for dispersed viruses, but a less powerful disinfectant is better in case of aggregates*.

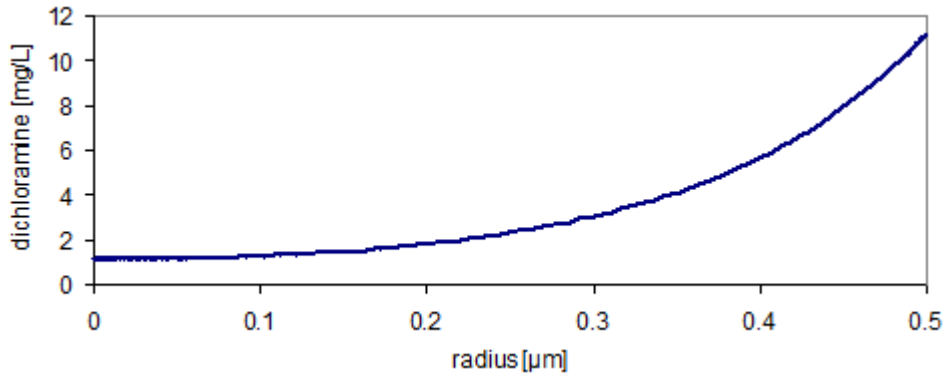

Figure 18: concentration of dichloramine inside aggregates of radius 0.5µm for an outer concentration of 11.1 mg/L.

Comparing the results obtained with PAA and dichloramine, the model seems to capture the inactivation of MS2 by both disinfectants. The differences of the fitted parameters (α = 0.7 instead of 0.8, and $\sqrt{k_2 / D}$ =9µm instead of 6.3 µm) could be the consequences of the small number of experiments performed with dichloramine, or of different dispersed disinfection law and absorption of disinfectant by the viruses.

3.3 PhiX174 inactivation by PAA

PAA as a disinfectant is well studied: it is relatively pH independent and stable, therefore more easy to work with than chloramines or chlorine.

3.3.1 Effect of pH on inactivation

As reported in the literature, we observed from experiments conducted at dispersing conditions (200 mM phosphate) that PAA was pH independent between pH 5 and 6. (Figure 19). The inactivation curves at both pH values looked the same. However, an important tailing was observed after several logs of inactivation, which was not observed with MS2. The tailing phenomenon goes beyond the scope of this work and was not further investigated.

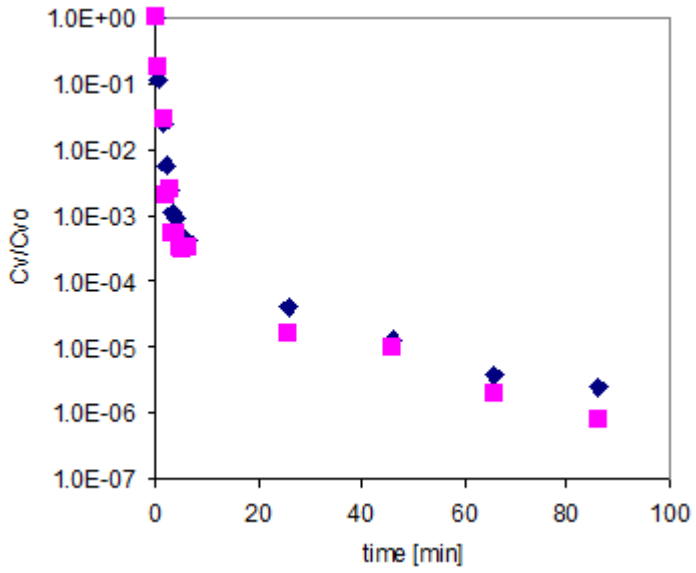

*Figure 19: Inactivation of dispersed PhiX174 (2*10^{10}pfu/mL) by PAA at pH 5 (16.1mg/L)in blue and 6 (19.1mg/L) in pink both at high phosphate concentrations (200 mM)*

3.3.2 Effect of aggregation on inactivation

In contrast to MS2, for PhiX174 no aggregation effect could be observed for the disinfection with PAA (see figure 20).

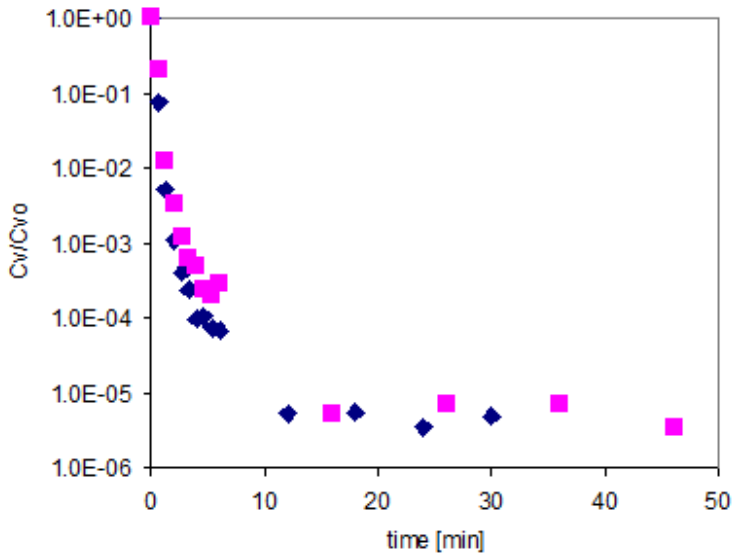

*Figure 20: Inactivation of aggregated PhiX174 (2*10^{10}pfu/mL) (pH 5, 15 mM phosphate,21.24 mg/L, in blue) and dispersed (pH 6, 15 mM phosphate,19.2 mg/L, in pink)*

The different shapes of PhiX174 and MS2 could explain the differences in susceptibility of inactivation to aggregation (see Figure 1). On the surface of PhiX174 little spikes on the capsid can be observed. These spikes may hinder compact aggregation (larger void spaces between adjacent viruses) and allow rapid diffusion of PAA into the aggregates. In the case that diffusion is much faster than disinfectant consumption, aggregation has no impact on disinfection.

Hence, we found another important factor concerning the impact of aggregation on disinfection: *The aggregate compactness depends on viral structure and determines a virus' susceptibility to aggregation effects on inactivation.*

3.4 PhiX174 inactivation by free Chlorine

Due to its high reactivity, disinfection experiments with free chlorine are very difficult to conduct. Especially at low pH values, where its high reactivity is maximal, chlorine consumption is very fast. Additionally, chlorine becomes unstable at pH values below 5.0. Even though PhiX174 aggregated at pH 5.0, it was still inactivated too quickly to allow inactivation studies (see Figure 21). Either the viruses were inactivated too quickly, or the free chorine was consumed too quickly to allow free chlorine concentration measures.

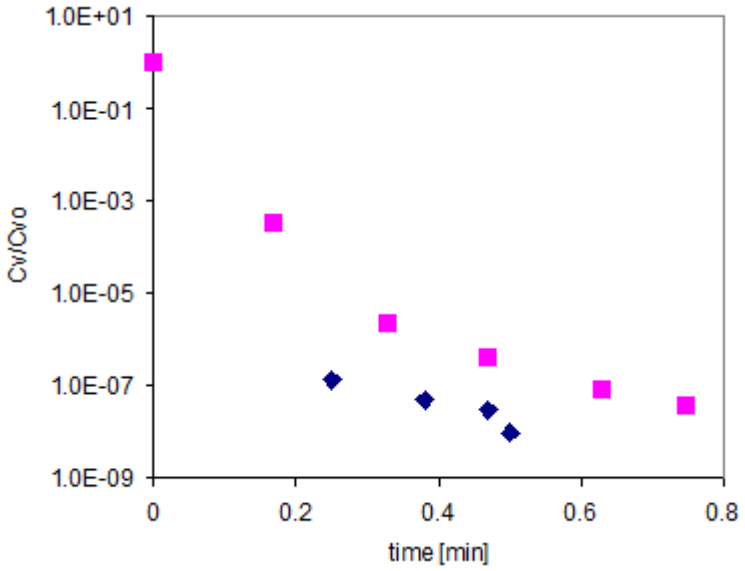

Figure 21: PhiX174 inactivated by free chlorine at pH 5 (blue) and pH 6 (pink) (pH 5 values are below detection limits)

4 Conclusions:

4.1 Disinfectants:

We have used different kinds of disinfectants in this project. It is interesting to compare them from an efficiency or an applicability point of view:

For drinking water disinfection, the most commonly used disinfectant is free chlorine. Free chlorine can be used as primary or secondary disinfectant[25]. The main advantages of chlorine are its strong efficiency, its simplicity of use and its price[37]. Disadvantages are predominantly by-product formation[38], high reactivity that does not allow long term stability, its taste and finally odor problems that can occur[25]. However, by-products are not a restraining factor for disinfection: a high concentration of remaining microorganisms in drinking water would be more dangerous than by-product formation[39]. In developing countries where cost can be an important factor chlorine can be an attractive disinfectant[40].

Our experiments and tests showed us that free chlorine is a very reactive and very strong disinfectant even at low concentration. Based on model predictions, we therefore expect this disinfectant to be drastically inhibited by aggregates. This may lower its appeal as a disinfectant. Due to its high reactivity at low pH, however, inactivation of aggregated viruses could not be experimentally assessed. In order to understand the impact of aggregation on disinfection by free chlorine, other viruses need to be found which aggregate at pH values higher or equal to 6.

Another widely used disinfectant of drinking water is chloramine. Chloramine is essentially used as secondary disinfectant[19]. In comparison to chlorine, chloramine is less reactive. It could therefore persist longer in the water with fewer degradation effects[30]. After using chlorine as primary disinfection, we can use chloramine as secondary disinfection for a long term effect in the water network (to avoid microbial re-growth). It permits to decrease the bad taste of water and induces fewer by-products than free chlorine secondary disinfection[19]. In terms of virus inactivation, our experiments confirm the results of previous studies; chloramines and especially monochloramine are much less effective than chlorine. Among the different chloramines, only monochloramine is used for disinfection, while the other species (di/trichloramine) are not desired[30]. Our results, however, indicate that inactivation of MS2 by dichloramine was faster than by monochloramine. The question therefore is: why do we not use dichloramine instead of monochloramine in drinking water disinfection process?

First monochloramine is stable at neutral pH in contrast to dichloramine: this property makes it easier to use and allow to know easily the concentration over time. Secondly, dichloramine is more dangerous for health (more irritant for eyes and nose, can cause stomach discomfort and risk of anemia)[41]. Finally, studies showed that dichloramine was less effective on bacteria[23,30], but contradictions still exist. If we want to use dichloramine in the disinfection process, future studies are needed, and additional training for the water treatment operators will be required.

4.2 Impact of viral aggregation on inactivation and its consequences on drinking water and wastewater disinfection:

Previous studies showed aggregation effect on inactivation. Our study confirms this point. We have found that compared to disperse viruses, inactivation of MS2 by dichloramine was slowed up to 13 times for aggregates of 600 nm radius. Our results confirmed those presented in the study of *Mattle et al, 2011*[14]: aggregation effects depend clearly on: the size of aggregates, the concentration and reactivity of this disinfectant.

We further found that weak aggregation like with PhiX174 viruses does not induce an aggregation effect on disinfection. In environmental conditions favouring aggregation, some kinds of viruses have stronger aggregation than other, though it is difficult to understand why. We can hypothesize that the shape or other specific virus conditions are the reason for this difference.

Aggregated viruses are common in the environment. Furthermore, inactivation of viruses in wastewater treatment plants is not complete, but varies between 60-99.9%[3,42], depending on viruses and conditions (type of treatment, time of treatment, environmental conditions). This suggests that some viruses able to aggregate at neutral pH could be found in drinking water treatment plants. Studies in order to determine the appropriate concentration of disinfectant in drinking water treatment plant are only done with dispersed viruses. Therefore in the case where these viruses are in strong and big aggregates the disinfection process could be insufficient.

4.3 Future work

Our study shows the impact of viral aggregation on inactivation. In some cases these aggregates strongly slow down the process and could be very problematical in terms of health

effects. Aggregation power depends on many parameters, for example, the ionic strength, the pH, the concentration of viruses and the type of viruses.

Further research is needed in order to find the types of viruses able to aggregate at neutral pH. They will highlight if a majority of problematic viruses for human are able to aggregate at neutral pH and if their aggregation is strong enough to have an impact on inactivation.

Experiments with other surrogate viruses able to aggregate strongly will be of great interest. This will allow to know if, with same disinfectant, the same results are obtained – thus if our results can be generalized across viruses. We furthermore need to find surrogate viruses able to aggregate pH values greater than 6 to be able to use free chlorine, because free chlorine is one of the most commonly used disinfectants in the world.

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6 Appendixes

6.1 Table of results MS2 and dichloramine

virus	concentration Cv [pfu/mL]	disinfectant	disinfectant concentration Cd [mg/L]	phosphate [mM]	pH	aggregate size [nm]	kapp [1/min]
MS2	5.E+10	d	7.35	15	3	425.5	0.391
	5.E+10	i	6.1935	15	3	455	0.405
	5.E+10	c	3.7825	15	3	421	0.216
	5.E+10	h	12.806	15	3	410	0.527
	1.E+11	l	5.5935	15	3	587	0.11
	5.E+10	o	7.332	400	3	-	0.537
	5.E+10	r	7.044	400	3	-	0.523
	5.E+10	a	6.9065	15	4	-	0.253
	5.E+10	m	7.356	15	4	-	0.322
	5.E+10	i	11.7745	15	4	-	0.245
	5.E+10	n	3.525	15	4	-	0.135
	5.E+10	e	6.1445	400	4	-	0.111

6.2 Organisms and Culturing methods

We used E. coli host for MS2 and PhiX174 plating. The infective concentration was observed with the double-layer agar method[27], and reported in pfu. Viruses are stored in PBS 5 mM phosphate 10 mM NaCl solution and conserved in the fridge.[14]

6.3 Buffers protocols

Thiosulfate solution

Goal: quench the disinfectant with 350 mg/L sodium thiosulfate solution

- put 35 mg of sodium thiosulfate in a flask
- add 100 mL of mili-Q water
- put the content in an autoclaved bottle

PBS buffer (low (5 mM) and high (150 mM) phosphate)

Goal: dilute viruses

- put 1.79g for low phosphate / 53.7g for high phosphate of Na_2HPO_4 $12H_20$ (for have 5 mM of phosphate) in a flask
- add 10 mL of NaCl 1Molaire (for have 10 mM of NaCl)
- complete with mili-Q water to have a little bit less of 1L
- adjust the pH at 7.5 with NaOH
- complete with mili-Q water to have 1L
- put the content in an autoclaved bottle
- autoclave the bottle with the solution

Buffer phosphate (low (15 mM) and high (200 or 400 mM) phosphate)

Goal: buffer where the experiment happens with the needed pH

- put 0.5171g (15 mM) / 6.8995g (200 mM) / 13.799g (400 mM) of $NaH_2PO_4H_2O$ in a flask
- add 3.75 mL of NaCl (15 mM)
- complete with a little bit less of 250 mL mili-Q water
- adjust the pH with HCl or NaOH (for low pH: add less of NaCl in proportion of HCl added (calculate with Phreeqc to have 15mM chloride at the end), if chloride to add with HCl exceeds 15mM, use H_3PO_4 instead HCl and make the deduction with the phosphate)
- complete to have 250 mL (re-adjust the pH if needed)
- filter the content at 100 nm
- put the content in an autoclaved bottle

KI solution 31.4 g/L for PAA measurement and 1 g/L for dichloramine measurement
Goal: give the colour for the KI colorimetric method or DPD colorimetric method

- add 3.14 g or 0.1 g of potassium iodide (KI) in a flask
- complete with mili-Q water to have 100 mL
- put the content in a dark bottle to avoid degradation

Buffer for KI colorimetric method
Goal: buffer for PAA measurement (add with KI solution)

- put 12.817 g of sodium acetate
- complete with a little bit less of 250 mL with mili-Q water
- adjust the pH to 5.4
- complete to have 250 mL
- put the content in an autoclave bottle

EDTA (Ethylenediaminetertraacetic acid) (different concentration depend of the use)
Goal: avoid bad reaction during experiments or measurement

DPD solution (concentrated / diluted)
Goal: give the colour for DPD colorimetric method (chlorine and dichloramine measurement)

- put 0.25 g of N,N-Diethyl-p-Phenylenediamine Oxalate in a flask
- put 1.5 mL mili-Q water with 0.5 mL H_2SO_4 (99%) in a tube
- add 50 mg EDTA in the tube (and mix)
- put the content of the tube in the flask and mix
- complete with mili-Q water to have 250 mL
- put the content in an autoclaved dark bottle to obtain the concentrated solution

- take 1.2 mL of the concentrated solution in a tube
- add 8.8 mL of mili-Q water to obtain diluted solution (re-do the diluted solution each day)

Buffer solution for DPD (concentrated / diluted)
Goal: buffer for DPD colorimetric method

- put 6.05 g Na_2HPO_4 $12H_2O$ in a flask
- add 4.6 g KH_2PO_4
- add 80 mg EDTA
- complete with 100 mL of mili-Q water
- put the content in an autoclaved bottle to obtain the concentrated solution

- take 1 mL of the concentrated solution in a tube

- add 9 mL of mili-Q water to obtain diluted solution

Potassium permanganate solution
Goal: calibration for DPD colorimetric method
- put 222.75 mg $KMnO_4$ in a flask
- complete with 250 mL of mili-Q water
- dilute 0.25 mL / 0.75 mL / 1.5 mL / 3mL of the solution to obtain 25mL with mili-Q water (correspond to 1 mg/L / 3 mg/L / 6 mg/L / 12 mg/L of chlorine for calibration)

6.4 Disinfectants protocols

Dichloramine stock (2.2 mM chlorine and Cl_2 / NH_4 = 4)
- put 14.9 mg NH_4Cl in a flask
- add 100mL mili-Q water
- add 0.092 mL of NaOCl (14%)
- put the content in a dark bottle
- wait 20 minutes (in mixing)
- low the pH at 4 (in mixing)
- wait 2h (in mixing) (you have to frequently re-adjust the pH) before using
- use in the 4 next hours

PAA stock (5 g/L)
- put 50 mL mili-Q water in a flask
- add 820 µL peracetic acid (PAA) (32%) (with tips containing filter)

Free chlorine stock (3.14 mg/L)
- put 9.9 mL mili-Q water in a flask "A"
- add 0.1 mL of NaOCl (13%) in the flask "A" (result in 1570 mg/L)
- put 100 mL mili-Q water in a flask "B" treated with a 2 mg/L chlorine bath
- add 0.2 mL of the flask "A" in the flask "B" (result in 3.14 mg/L free chlorine solution)

6.5 Measurements protocols

Free chlorine DPD colorimetric method
- all the material have to be treated with chlorine bath 2 mg/L (cuvette, and tips)
- put 0.05 mL of DPD diluted solution in a 1.5 mL cuvette
- add 0.05 mL of buffer for DPD (diluted)
- add 1 mL of sample
- take a measurement with the spectrophotometer at 515 nm using the calibration done with potassium permanganate solution
- divide the result by 10

PAA KI colorimetric method
- put 0.1 mL of KI solution (31.4 g/L) in a 1.5 mL cuvette
- add 0.8 mL of buffer for KI
- add 0.1 mL of sample

- take a measurement with the spectrophotometer at 352 nm (find the concentration with the law: $A = \alpha \cdot C \cdot l \rightarrow C = \frac{A}{\alpha \cdot l}$ with α = 24100 L/(mol*cm)

Dichloramine direct absorbance method

- put 1 mL of the sample in a cuvette
- take a spectrum 200-600nm with the spectrophotometer
- take the peaks at 254 nm (monochloramine) and 295 nm (dichloramine)
- convert in concentration with the calculation describe in Cimetière 2009 thesis[21]

$$[NH_2Cl] = \frac{\frac{A_{254\,nm}}{\varepsilon_{NH2Cl,254}} - \frac{\varepsilon_{NHCl2,254} \times A_{294\,nm}}{\varepsilon_{NH2Cl,254} \times \varepsilon_{NHCl2,294}}}{1 - \frac{\varepsilon_{NHCl2,254} \times \varepsilon_{NH2Cl,294}}{\varepsilon_{NH2Cl,254} \times \varepsilon_{NHCl2,294}}} \qquad [NHCl_2] = \frac{\frac{A_{294\,nm}}{\varepsilon_{NHCl2,294}} - \frac{\varepsilon_{NH2Cl,294} \times A_{254\,nm}}{\varepsilon_{NH2Cl,254} \times \varepsilon_{NHCl2,294}}}{1 - \frac{\varepsilon_{NHCl2,254} \times \varepsilon_{NH2Cl,294}}{\varepsilon_{NH2Cl,254} \times \varepsilon_{NHCl2,294}}}$$

avec $\varepsilon_{NH2Cl,\,254} = 369\ M^{-1}\ cm^{-1}$ $\varepsilon_{NH2Cl,\,294} = 15\ M^{-1}\ cm^{-1}$

$\varepsilon_{NHCl2,\,254} = 136\ M^{-1}\ cm^{-1}$ $\varepsilon_{NHCl2,\,294} = 289\ M^{-1}\ cm^{-1}$

Dichloramine DPD colorimetric method

- put 0.5 mL of DPD concentrated solution in a 1.5 mL cuvette
- add 0.5 mL of buffer for DPD (concentrated)
- add 0.1 mL of sample
- take a measurement with the spectrophotometer at 515 nm using the calibration done with potassium permanganate solution (FRC measurement)
- add 1 mL of KI solution (1 mg/L)
- take the measurement after exactly 2 minutes 515 nm using the calibration done with potassium permanganate solution (TRC measurement)
- concentration of chloramines CRC = TRC - FRC

6.6 Experiments protocols

MS2 by dichloramine

- Poor 500µL of 15mM phosphate buffer with the desired pH (3 or 4) and the desired phosphate concentration (15 mM or 400 mM) into a plastic cuvette
- Add 10µL of virus stock solution to the buffer solution
- Start DLS player of the DLS unit
- Let sample aggregate 1h (only for pH 3 15 mM phosphate)
- Add the desired quantity of dichloramine stock solution at pH 4 in the phosphate buffer with the desired pH and re-adjust to the desired pH (we take 1/5 dilution for have about 7 mg/L)
- Measure concentration using UV spectrum (with the both methods)
- Take sample of the solution with MS2 at t=0min (10µL sample into 240µL thiosulfate solution and 240µL PBS (5 or 150 mM depending of the buffer phosphate))
- Add 500µL of dichloramine diluted at the needed concentration with the buffer, in the solution with virus, (time zero) very gently!
- Measure concentration using UV spectrum (as fast as possible) (DPD method)
- Take samples at different time (20µL sample into 240µL thiosulfate solution and 240µL PBS (5 or 150 mM depending of the buffer phosphate)
- Measure concentration using UV spectrum (at the end)
- Measure pH after experiments

PhiX174 by PAA

- Add 15µL of 2mM (for 15mM phosphate) / 20mM (for 200 mM phosphate) EDTA solution into 15mM phosphate buffer at desired pH (5 or 6) with desired phosphate concentration (15 or 200 mM)
- Add 40µL (less or more for the desired concentration) of 5g/L PAA solution into 5mL of buffer solution with EDTA re-adjust to the desired pH
- Poor 250µL of phosphate buffer with EDTA at the desired pH into 1.5mL plastic cuvette
- Add 5µL virus stock solution in the 250 µL
- Start DLS player of the DLS unit
- Let sample aggregate 1h (only for pH 5 15 mM phosphate)
- Take a sample (t=0) 5 µL of solution (put in 200/200 µL thiosulfate and PBS (5 or 150 mM depending of the buffer phosphate))
- Add 750µL of PAA with buffer solution (time zero) very gently!
- Measure PAA concentration using UV spectrum
- Take samples at different time (40µL sample into 200µL thiosulfate solution and 200µL PBS (5 or 150 mM depending of the buffer phosphate))
- Measure PAA concentration using UV spectrum
- Measure pH after experiments

PhiX174 by free chlorine

- Use materials (tips, tube, cuvette) treated in chlorine bath
- Add 25µL (less or more for change concentration) of Chlorine stock solution into 10mL of phosphate buffer at desired pH, readjust the to desired pH and wait 10minute before experience to stabilize the concentration of chlorine
- Mesure chlorine concentration at 5 and 10 minute for see if stable (DPD colorimetric method)
- Poor 900µL of buffer solution with desired pH into 1.5mL plastic cuvette (with tips and cuvette not treated!)
- Add 9µL of virus stock solution into the cuvette (use not treated tips)
- Start DLS player of the DLS unit
- Let sample aggregate 1h (only for pH 5 15mM phosphate)
- Add 4mL of Chlorine and buffer solution (stable 10-30+min) in an other plastic cuvette for each different concentration
- Measure Chlorine concentration using UV spectrum (1 times) of the plastic cuvette (rest 3mL)
- Take a sample (t=0) of the solution of virus with buffer (200µL sample into 200µL thiosulfate solution and 200µL PBS (5 or 150 mM depending of the buffer phosphate))
- At t=-10 take 0.300mL of virus and buffer solution in a 1mL tips (treated tips wash with buffer)
- At t=0 put the virus and buffer solution in the cuvette containing buffer and chlore solution
- Take samples at different time (200µL sample into 200µL thiosulfate solution and 200µL PBS (5 or 150 mM depending of the buffer phosphate))
- Measure Chlorine concentration using UV spectrum (1 times)
- Measure pH after experiments