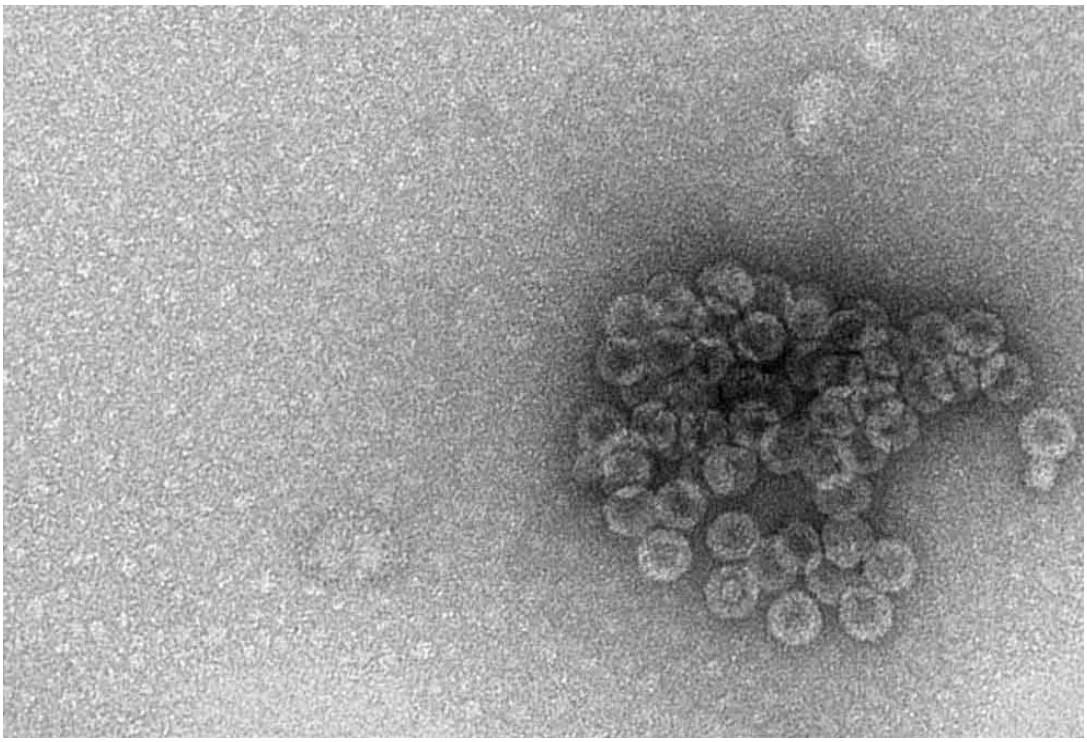
Disinfection Kinetics of Virus Aggregates of Bacteriophage MS2



Master Thesis

Moritz Brennecke

Supervised by:

Prof. Tamar Kohn

Assisted by:

Krista Wigginton

Laboratory:

Laboratoire de Chimie Environnementale (LCE)

Ecole Polytechnique Fédérale de Lausanne

June 2009

Acknowledgements

I would like to thank everybody who helped me with this work. Especially I would like to thank Krista Wigginton, for correcting my work, helping me to design the experiments and giving psychological support. Tamar Kohn for providing me with literature and knowledge and guiding me through my thesis. Davide Demurtas for sacrifying his time to supply me with wonderful MS2 images. Luisa Valério Martin for helping out in the laboratory whenever there was a problem. Francois Mayrte for helping me along with the DLS instrument and the treating of DLS data. Finally I would like to thank my laboratory for making the work on the master thesis fun.

CONTENTS

1 Abstract

The aim of this study was to determine the difference in disinfection between aggregated and dispersed virus. Bacteriophage MS2 was chosen as a surrogate for enteric virus. The first part of this project consisted of characterizing the aggregation of MS2 and the second part examined differences in the disinfection of aggregated and dispersed virus solutions.

It was shown that MS2 only aggregated at a pH below its isoelectric point when the solution ionic strength was below 50 mM. At neutral pH, high ionic strength and $CaCl_2$ did not cause aggregation. High ionic strength showed inhibition of aggregation at low pH. Aggregation was also influenced by the choice of buffer. The effect of the solution chemistry on the extent of aggregation at pH 3 was the following: acetic acid (20 mM)>sodium chloride (20 mM)> phosphate buffer (5 mM PO_4^{2-}, 10 mM NaCl).

Disinfection of the virus was thus tested at a pH above and under the isoelectric point at low ionic strength (phosphate buffer and 20 mM NaCl) in order to have dispersed and aggregated MS2 virus, respectively. Ultraviolet light (UV) and peracetic acid (PAA) were the chosen disinfection techniques as they are generally regarded as pH independent. UV showed greater disinfection capacity for the aggregated system than for the mono- dispersed system. Both disinfection curves showed first order kinetics. This observed difference in disinfection rates may not be due to the aggregation state of the virus but to other mechanisms, including the effect of pH on direct and indirect oxidation (by ROS) of amino acids in the virus capsid proteins. This is supported by the fact that UV can penetrate within the virus unlike a chemical disinfectant which must be in physical contact with a virus component for a reaction to take place. In order to test the effect of direct contact by chemical disinfectants, an aggregated and a dispersed solution were spiked with PAA. A difference in the behavior of the disinfection curves in phosphate buffer was observed after an inactivation of 6 logs. The disinfection rate of the aggregated system slowed down (tailing) after some initial amount of disinfection, while the dispersed system was completely disinfected with one disinfection rate. This implies a solution with some large aggregates and many single viruses in the solution. In 20 mM NaCl the disinfection rate of the aggregated virus was much slower than for the dispersed system ($k_{aggregated}$= 0.0224; $k_{dispersed}$= 0.0747). Both inactivation curves showed first order kinetics. By applying Grant's model (1995) an aggregation factor was calculated (b= 1.413). This indicates a very aggregated state with large aggregates.

Résume en français

L'objectif de cette étude était de déterminer la différence entre la désinfection de virus agrégés et dispersés. MS2 a été choisi comme un substitut de virus entériques. La première partie a consisté en l'agrégation de MS2, alors que la deuxième partie a traité de la désinfection d'agrégats de virus et de virus dispersés. Il a été démontré que MS2 s'agrège uniquement à un pH plus bas que son point isoélectrique et à une force ionique inférieure à 50 mM. Une force ionique élevée, la présence de $CaCl_2$ et un pH neutre ne provoque pas d'agrégation. De même, il a été montré qu'une force ionique élevé et un pH faible entraine une inhibition de l'agrégation à faible pH. L'agrégation est également limitée par le choix du tampon. Il a été montré que l'agrégation est plus forte dans les conditions suivantes: acide acétique (20 mM)> chlorure de sodium (20 mM)> tampon phosphate (5 mM PO_4^{2-}, 10 mM NaCl). La désinfection a donc été testée à un pH au-dessus du point isoélectrique à faible force ionique (tampon phosphate et 20 mM NaCl) dans le but d'obtenir les virus MS2 sous forme dispersés et agrégés. L'acide acétique a été écarté car il s'agit d'un composé organique qui donc réagir avec les désinfectants utilisés dans les expériences. Deux techniques de désinfection (UV et acide peracétique [AAP]) dont l'efficacité de la désinfection n'est pas fonction du pH ont été choisies. La désinfection aux UV a montré une plus grande capacité à faible pH pour le système agrégé que pour le système mono-dispersé. Les deux courbes montrent des cinétiques de désinfection du premier ordre. Il a été suggéré que cet effet n'est pas dû à l'agrégation, mais à d'autres mécanismes, y compris l'oxydation directe et indirecte (par les dérivés réactifs de l'oxygène) à cause du changement de la structure des acides aminé par la protonation. Cela est corroboré par le fait que l'UV ne nécessite pas de contact direct avec le virus pour réagir. Afin de tester l'effet d'un contact direct par des désinfectants chimiques, l'AAP a été ajouté à une solution agrégée et une solution dispersée. Dans le tampon phosphate un comportement différent de la courbe de désinfection a été observé après une inactivation de 6 logs. La désinfection de l'agrégat a ralenti, alors que le système dispersé a été complètement désinfecté. Cela implique une solution avec quelques agrégats de grande taille et beaucoup de virus mono- dispersé. Dans 20 mM de NaCl, la désinfection du virus agrégé a été beaucoup plus lente que pour le système dispersé ($k_{agregé}$ = 0,0224; $k_{dispersé}$ = 0,0747). Les deux inactivations montraient une cinétique de premier ordre. En appliquant le modèle de Grant (1995) un facteur d'agrégation a été calculé (b = 1,413). Cela indique un état fortement agrégé avec de grands agrégats.

2 Introduction

Viruses are ubiquitous in the environment (Kutter and Sulakvelidze 2004). Although most viruses are not pathogenic, many are known to harm humans (measles, HIV). A recent example of a virus that causes a serious health threat to humans is the swine flu outbreak across the globe. The swine flu, like other influenza viruses, is believed to be transferred mostly by the respiratory route. Virus transmission by water is also common. For example, enterovirus species are a major cause of gastroenteritis. Additional human viral diseases that spread through water include hepatitis A and Poliovirus. Water is thus commonly disinfected to avoid the spread of viral and other human waterborne diseases. Efficient disinfection of virus in water depends on water conditions, the disinfectant, the type of virus, and also on the aggregation state of viruses (Thurman and Gerba 1988).

The aggregation of individual virus particles is common in the environment (Narang and Codd 1981, Sharp et al. 1975, Galasso and Sharp 1962, Young and Sharp 1977). Improved knowledge about virus aggregation can help to predict the structure of the virus and its interactions with other material in its environment (adsorption, filtering). It will also aid in optimizing the design of water treatment techniques such as filters and disinfection doses to remove aggregated viruses. Furthermore, understanding the interactions between viruses will help the handling of viruses in the laboratory and avoid false interpretation of results, caused by aggregation of viruses.

Disinfection kinetics depend not only on the environmental conditions (pH, Temperature, etc.), but also on the aggregation state of the virus (Galasso and Sharp 1965). Few studies have clearly shown the differences in reaction kinetics between dispersed and aggregated viruses in experimental studies (Sharp et al. 1975, Floyd and Sharp 1977, Galasso and Sharp 1965, Thurston-Enriquez et al. 2003). Grant (1995) summarizes these works and concluded that there is a tendency for aggregates to be more difficult to disinfect than mono-dispersed solutions. Unfortunately, most of the studies available on the subject date back to the 1970's and no recent studies have been conducted, despite the fact that disinfection kinetics are of high importance to assure drinking and wastewater quality standards.

In this study, MS2 was chosen because it is often used as a surrogate for human enteric viruses. It has the same size and disinfection properties as other enteroviruses (Garbow et al 1984). Additionally, MS2 is easy to handle and is not pathogenic.

The aim of this study included the following:

- identify the factors that influence the aggregation of MS2
- define the aggregate size
- analyze the disinfection kinetics of aggregated vs. mono- dispersed solution

2.1 Virus aggregation

In nature, viruses often form aggregates. Aggregates in the environment are difficult to find because they are in such low concentrations. However it is suspected that these aggregates exist because they are present in the feces of persons infected by virus (Teunis et al. 2008, Narang and Codd 1981). Hejkal et al. (1981) identified that after sonication of samples from a water treatment plant, the number of PFU increased. He thus concluded that the viruses were associated in aggregates or absorbed onto solids. Young and Sharp (1977) showed that aggregation of Poliovirus occurs after lysis of the host cell and remained aggregated in finished water and reservoir water of a treatment plant. Furthermore, virus aggregation has been observed under varying solution chemistry (Mayo and Roberts 1979, Sharp et al. 1975, Floyd and Sharp 1977, 1978, 1979, Jensen et al. 1980, Galdiero 1979, Abad et al. 1994, Kim and Sharp 1966, Langlet et al. 2008b).

A major difficulty with analyzing virus aggregates is the lack of techniques available capable of differentiating mono-disperse from aggregated virus. At this time, the most common technique to detect and quantify virus is by the Plaque Forming Units (PFU) technique (also "plating"). Briefly, virus is diluted into a countable number, mixed with its host, spread as a monolayer, incubated to allow virus infection of the host cells, and then the resulting infections of the host cells are counted. A problem with this technique is that either an aggregate of virus or a single virus produces one PFU. Thus no differentiation can be made between single viruses and virus aggregates (Galasso and Sharp 1962). Consequently other techniques are necessary to supplement the plating results in order to understand the presence and size of aggregates.

A number of methods have been employed to induce and study aggregation for laboratory aggregation studies. Floyd and Sharp (1977) reported the aggregation of Poliovirus and Reovirus at acidic pH. The viruses also aggregated in distilled water. They used the single particle approximation (SPA) test, a method which separates aggregates by ultracentrifugation and confirmed results with Transmission Electron Microscopy (TEM). Langlet et al. (2007

and 2008b) observed a reduction of PFU with MS2 when lowering the pH under the isoelectric point. They verified their result by combining results of dynamic light scattering and flow particle image analysis (FPIA). Floyd and Sharp (1978a) also analyzed the influence of salts on the aggregation of Poliovirus and Reovirus with SPA and TEM. Aggregation was induced by lowering the pH. Divalent ions like calcium and magnesium played an important role in aggregation, but at different concentrations they can either intensify or inhibit aggregation. The most important factors influencing the aggregation are apart from the virus type, pH, ionic strength and the composition of ions.

All three factors (ionic strength, divalent Ions and pH) were investigated in this study to induce aggregation of MS2. The methods used to identify aggregates were TEM and DLS, because these techniques are both well established and are available at the institute. Cryo-TEM and FFF (Costello 2006, Chuan et al. 2008) are additional techniques for virus analysis, but were not available for this study.

The theoretical approach of the aggregation state of a system of particles is given in the following section.

Zeta potential and electrophoretic mobility

The zeta potential is the potential of the particle's electric potential in the electric double layer. It describes the potential difference between the point at which ions still travel with the particle (slip plane) and the medium (Delgado et al. 2005). The stability of a system of colloids can be described by the zeta potential: If the potential is largely negative or positive, the particles will repulse each other and stay dispersed. If the potential is near zero (typically considered ±20mv) the system will collapse and particles will aggregate. The main influences are the pH and the ionic strength of the solution (Delgado et al. 2005). Thus, by changing the ionic strength or the pH one can favor the aggregated state of the viruses.

The zeta potential is linked and measured by the electrophoretic mobility of a particle system. Electrophoresis is the mobility of the particles under an applied electric field. The electrophoretic mobility is measured by dividing the speed of particles by the electric field strength, where

$$U_e = \frac{v}{E} \qquad (1)$$

U_e = electrophoretic mobility [$m^2 s^{-1} V^{-1}$]

ν = velocity [$m s^{-1}$]

E = electric field applied [$V m^{-1}$]

The electrophoretic mobility is related to the zeta potential by:

$$U_e = \frac{2\varepsilon_0 \varepsilon_r \zeta f(\kappa a)}{3\eta} \quad (2)$$

ε_0 = electric permittivity of vacuum [$F m^{-1}$]

ε_r = relative permittivity of the dispersion medium

ζ = zeta potential [V]

$f(\kappa a)$ = Henry's function (describes the model for the double layer)

η = dynamic viscosity [Pa s]

a = radius of the particle [m]

κ = = reciprocal Debye length[m^{-1}]

The κ in Henry's functions stands for the double layer thickness. It can be calculated by

$$\kappa^{-1} = 10^{10}\left[\frac{2000e^2 N_A I}{\varepsilon\varepsilon_0 kt}\right]^{-1/2} \quad (3)$$

NA = Avagadro's number [mol^{-1}]

I = ionic strength [$mol\ m^{-3}$]

k = Boltzmann constant [$J K^{-1}$]

T = absolute temperature [K]

e = elementary charge [C]

ε_0 = permittivity in a vacuum [$F m^{-1}$]

ε = permittivity of water

Equation 3 shows that he thickness of the double layer and thus the stability of a system depends on the ionic strength. Divalent or trivalent ions will reduce the double layer even faster (Crittenden et al. 2005). Divalent ions can get closer to the virus (they have greater charge) and shield its overall charge. Trivalent ions are even more effective, but were not used in this work, because aluminum is known as a biocide and iron precipitates very easily, so differentiation between adsorption to precipitates and pure virus aggregates would be difficult.

Calcium Chloride ($CaCl_2$) is more effective than other bivalent ions, because it is very small and can get closer to the virus. Furthermore, it has no known toxicity to virus at high concentrations.

In practice, the determination of the real zeta potential of a particle, where diffusion inside the particle coat plays a major role (organic molecules or "soft particles"), is difficult to model. Ohshima (2007) first introduced a model of a soft particle. It contained a hard core and an ion-penetrable surface layer of polyelectrolytes. Langlet et al. (2008a) extended this model for the MS2 virus by introducing three different layers: a bulk RNA region (~8 nm), an RNA protein bound layer (~3 nm) and a proteic capsid shell (~2 nm). Even with their complex mathematical model, the resulting prediction for the electrophoretic mobility and thus the stability of the system were not accurate for low pH solution.

For simplicity and the lack of accurate models, a "hard particle" approximation was used in this work. A hard particle is a particle with a hard cover and cannot be penetrated by salts.

Ohshima (1994) derived a simple equation for calculating Henry's function of a hard particle:

$$f(\kappa a) = 1 + \frac{1}{2}\left[1 + \left(\frac{2.5}{\kappa a\left[1 + 2\exp(-\kappa a)\right]}\right)\right]^{-3} \qquad (4)$$

The isoelectric point (pI or point of zero charge) is easier to determine. This is the point where no electrophoretic mobility is measured and hence the particle is without any charge. It is the point where the system is instable because there are no more repulsive forces.

Due to the structural proteins of MS2, it has a global negative charge at a neutral pH with a reported isoelectric point of 3.1- 3.9 (Redman et al. 1997, Zerda and Gerba 1984, Langlet et al. 2008b). By lowering the pH to the isoelectric point (pI), the virus can get to the point where it has no charge. Without any charge it will be hydrophobic and van-der-Waals attractive forces will become highly important.

2.2 Virus and disinfection

Aggregates are known to be more resistant to disinfection than mono-dispersed solutions (Grant 1995, Wallis and Melnick 1967). Infections can be caused by either a single virus or an aggregate. In theory, virus aggregates are disinfected only on the outside leaving infective viruses within the aggregate (Thurman and Gerba 1988). Young and Sharp (1977) observed significant differences in the disinfection of aggregated versus dispersed poliovirus by

bromine. Disinfection of aggregates resulted in a levelling off of the disinfection curve (tailing). Sharp et al. (1975) also reported the tailing effect when aggregated Reovirus was disinfected with bromine. They then treated the surviving units with sonication (20 khz) and this increased the titer 10-43 fold, thus aggregates could responsible for the tailing. TEM images did not reveal larger aggregates, but they did suggest the presence of some large aggregates not countable by electron microscopy. Thurston-Enriquez et al. (2003) compared poliovirus 1 and feline calicivirus inactivation by chlorine in aggregated and dispersed states. They used chloroform to produce a dispersed solution, but did not analyze and characterize the aggregated state. Their disinfection studies also showed significant levelling off for the aggregated viruses.

Kinetics

For kinetic experiments that examine the affect of a disinfectant concentration, pH and temperature are typically held constant as the time and concentration of the disinfectant are measured. Disinfectant concentration and disinfection time are multiplied to give the Ct value in the Chick's (concentration [mg*L^{-1}]*time [min]) which links the inactivation rate to the concentration of the disinfectant and the time (Chick 1908, Watson 1908).

$$\frac{dN}{dt} = -kC^n N \Rightarrow \ln(\frac{N}{N_0}) = -kC^n t \qquad (5)$$

N = concentration of organism [PFU/ml]

N_0 = initial concentration of organism [PFU/ml]

k = rate constant or coefficient of specific lethality [units vary with n]

C = concentration of the disinfectant [mg/L]

t = duration of exposure [s]

n = dilution constant

The constant "n" only exists when the disinfectant activity depends on its concentration. If this is not the case then time and concentration are equally important. In most cases, n= 1, and the reaction follows first order kinetics if the concentration of disinfectant is constant.

$$\frac{dN}{dt} = -kCN \Rightarrow \ln(\frac{N}{N_0}) = -kCt \qquad (6)$$

For ultraviolet (UV) inactivation, the model is modified with the UV-Dose or fluence. Here, the intensity replaces the concentration in the Chick- Watson model.

$$\ln(\frac{N}{N_0}) = -kIt \quad (7)$$

I = intensity [W m^{-2}]

It = fluence [J m^{-2}]

Disinfection of aggregates

For inactivation kinetics of viral aggregates, Grant (1995) developed a model which involves probability of an aggregate to be infective:

$$p_j(\tau) = 1 - (1 - e^{-\tau})^j \quad (8)$$

τ = k*t (rate constant multiplied by the time)

j= aggregate of j viruses

The overall distribution of aggregates is linearly approximated with an aggregation factor b, which is the slope of the distribution curve in a double log plot (group size against frequency):

$$c_j(0) = j^{-b} c_i(0) \quad (9)$$

$c_i(0)$ = initial concentration of single viruses [PFU/ml]

b = aggregation factor

Every distribution is thus characterized by only one aggregation factor b. This factor normally varies between 4 for mono-dispersed and 1.3 for aggregated virus solutions. A major problem with Grant's model is that the aggregation factors were determined based on only a few transmission electron microscopy studies and most of them do not show aggregates of virus large enough to shield against disinfection. The effect of aggregation in disinfection is normally seen by aggregates of at least 16 virus particles (Thurman 1988). Smaller aggregates will not yield to shielding of the virus in the center.

Loss of PFU can then be described as:

$$Loss\ PFU = \frac{PFU}{PFU_0} = \frac{\sum_{j=1}^{\infty} p_j(\tau)c_j(0)}{\sum_{j=1}^{\infty} c_j(0)} \quad (10)$$

PFU_0= initial PFU

In practice, complete size distributions are often not available. Therefore Grant also suggested a simplified model. This model is linear on a semi log plot and thus does not take tailing into account. It is a variation of Chick's model:

$$\ln(\frac{N}{N_0}) = \frac{-kC}{\zeta(b)} \quad (11)$$

$\zeta(b)$ = Riemann zeta function of b
The zeta function results from the calculation of the slope b introduced in equation 9.

Disinfectants
For water disinfection, chlorine is the most common disinfectant used over the world, because it is easy to handle and cheap to produce. Clevenger et al. (2007) proved the effectiveness of chlorine in MS2 inactivation. Using hypochlorite, they showed a 4 log inactivation with a Ct-value of one. In Europe, chlorine dioxide (ClO_2) is also a very common water disinfectant. Thurston-Enriquez et al. (2005) tested chlorine dioxide on adenovirus (AD40) and calicivirus (FCV) and report a Ct-value for a 4 log virus reduction in the range of 0.12- 1.59 (AD40) and 0.18- 30.30 (FCV), respectively when pH and temperature ranges of 6-8 and 5- 15 ^oC were employed. Ozone is also often used as a chemical disinfectant. Gordon and Fairbairn (1991) found that an ozone residual of less than 40 µg/liter inactivated 4 logs of MS2 coliphage in 20 seconds. UV radiation is often used in water treatment since it was demonstrated that it is very effective against (oo)cysts of Cryptosporidium and Giardia, two pathogenic microorganisms with high resistance against chlorine. A 4 log disinfection of MS2 with a UV-fluence of 72 mJ/cm^2 is possible (Hijnen et al. 2005). A wide range of other chemical disinfectant exists. One of the emerging disinfectants is peracetic acid. It has no known disinfection by products (DBP) or toxic residuals commonly observed by treatment with halogen- containing disinfectants, and has a greater oxidation potential than chlorine (Kitis et al. 2004).
As one of the goals of this study is to induce aggregation by altering the solution pH, a disinfectant with pH dependence would interfere in the disinfection kinetics. Unfortunately

chlorine (the most common disinfectant used over the world) could not be used in this study due to its high pH dependency (Cherney et al 2006). Activities of ozone and chlorine dioxide are also pH dependent (Ershov and Morozov 2008, Crittenden et al. 2005, Thurston-Enriquez et al. 2005).

For this study we have chosen to work with peracetic acid and UV because these disinfectants are generally regarded as pH independent (Kibis et al. 2004, Hijnen et al 2006). Peracetic acid disinfects due to the release of active oxygen, like other peroxides (Kibis et al. 2004). UV disinfection includes two major processes: direct oxidation and indirect oxidation. Direct oxidation can cause dimerization of nucleotides of the DNA (or RNA) and thus inhibits reproduction (Crittenden et al. 2005). Direct oxidation damage is also known for amino acids (Davies 2003). Indirect oxidation of proteins is caused by the formation of reactive oxygen species (ROS), especially singlet oxygen, which then react with biomolecules within the organisms.

2.3 MS2

MS2 was chosen as a surrogate for other viruses, because it has a comparable size and disinfection kinetics as other enterovirus (Garbow et al 1984). MS2 belongs to the family Leviviridae. It is an icosahedral bacteriophage with a diameter of 29 nm (Boedtker and Gesteland 1975, Figure 1). In the laboratory, MS2 is typically propagated with Escherichia coli.

The MS2 genome encodes four proteins: 180 copies of a coat protein, one copy of an A-Protein (or maturation protein), which is involved in the folding of the virion (Kuzmanovic et al. 2006) and is possibly involved in the pilus recognition (Date 1979), a replicase for genome multiplication, and a lysis protein.

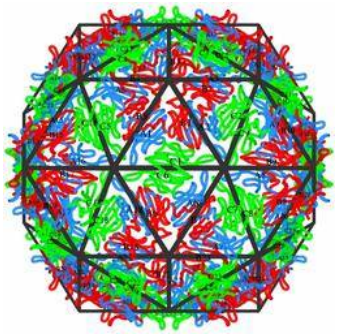


Figure 1: MS2 Cage (http://viperdb.scripps.edu/Cages/WhiteTrimmedScaled/2ms2_cage_white.jpg, access: 25 May)

3 Materials and methods

3.1 Preparation and treatment of samples

3.1.1 Materials and buffer

A detailed overview of the material is found in the appendix. Detailed information on plating and sterile conditions is also found in the appendix. Buffers were prepared with Milli Q-treated deionized water and autoclaved before using. For DLS measurements, buffers were filtered through a 100 nm filter (Millex-VV Syringe driven filter unit, Millipore). Phosphate buffer (PBS, 5 mM $PO_4$2-, 10 mM NaCl) was prepared using monobasic sodium phosphate (Acros organics). Carbonate Buffer (1 mM CO_3^{2-}, 10 mM NaCl) was prepared using sodium bicarbonate (Acros organics).

3.1.2 E.Coli and MS2

MS2 and its *E.Coli* Host were acquired from the German collection of Microorganisms and Cell Cultures (DSMZ Nos. 13767 and 5695, Braunschweig, Germany).

Growth of MS2: LB Broth was warmed to 40 ˚C in a water bath and 5ml of overnight culture of *E.Coli* was added and shake at 80 rpm. The optical density was measured spectrophotometrically until a value of 0.04 at 620 nm was reached. Then 10 µl of 10^{11} PFU/ml of the acquired virus stock was added and the culture was left for 4 hours at 37 ˚C without shaking. 5 ml chloroform ($CHCl_3$) was added to lyse the bacteria. After mixing $CHCl_3$ was left at least 15 minutes to settle. The chloroform was removed and air bubbled through the media to remove chloroform residuals. The aqueous phase was centrifuged for 15 minutes (3385*g) and the supernatant mixed with NaCl (0.5 M) and PEG (10 % w/v). The culture was left overnight at 4 ^{0}C. The broth was centrifuged for 45 minutes (10000*g) and the liquid phase removed. 3.5 ml PBS was added to the pellet and set to soak for 2- 12 hours. The pellet was placed in a 50 ml tube and brought up to 40 ml. It was then centrifuged for 5 minutes (10000*g). The supernatant was transferred to a new tube and 10 ml $CHCl_3$ added. The solution was mixed and centrifuged for 10 minutes (3000*g). The aqueous part was placed in a clean 50 ml tube. The procedure with $CHCl_3$ was repeated 4-5 times until the white emulsion between the $CHCl_3$ and aqueous phases was totally removed. After cleaning the aqueous phase was placed in a clean tube and centrifuged to settle residual $CHCl_3$. A 100 times concentration of the virus was prepared by passing the aqueous phase through an Utracell Centricon tube (100 kDa, Millipore, Billerica, MA) by 15-20minutes centrifugation

(3385*g). The suspension was washed three times in PBS. The final volume of approximately 750 µl was filtered through a 0.1 µm filter (Millex-W PVDF, Millipore).

3.1.3 Buffer exchange

Viruses stocks were stored in PBS. In many cases it was necessary to exchange the phosphate buffer prior to experiments to avoid problems like the precipitation of chemicals or to obtain a different ionic composition in the solution. This was conducted with 500 µl Microcon filters (Ultracel YM 100) in the Eppendorf centrifuge (5417R).

Briefly, the buffer exchange was performed by adding approximately 400 µl of the desired buffer to the top of the Microcon filter along with the desired amount of virus stock and was then centrifuged at 6000*g for 4 minutes, or until the volume in the top of the microcon was approximately 20 µl. 400 µl of buffer was added again to the top of the filter and the centrifugation step was repeated. The filter was then turned upside down into a new centrifuge tube and centrifuged at 1000*g for one minute to capture the virus.

3.2 Experimental setup

3.2.1 Dynamic Light Scattering (DLS)

Measurements were performed with the Zetasizer Nano ZS (Malvern Instruments) equipped with an autotitrator (mpt-2, Malvern Instruments) in disposable 120 µl cuvettes. The cuvettes were always placed in the instrument with the same orientation and care was taken to avoid air bubbles in the cuvettes. The program (Dispersion Technology Software 5.10, Malvern Instruments Ltd.) was set to 13 runs of 10 seconds in each measurement, because at this time, MS2 correlation data always stabilized. The measurement was repeated at least three times.

Parameters in the model were temperature (always set to 25^{o}C) and material (latex, only necessary for the calculation of number distributions). A brief description of the zetasizer theory is given in the appendix.

3.2.2 Transmission Electron Microscopy (TEM)

A Philips CM100 TEM with images taken at 8 0kV with a camera CCD 2kX2k was used to collect all TEM images. For TEM measurement preparations, 15 µl of sample was pipetted onto parafilm. A grid was put with the carbonated side on top of the sample drop and left there for about 30 seconds. Then the grid was transferred to the top of a drop of 500 µl of buffer where it was left for about 30 seconds. The water on top of the grid was wicked away

by touching the side of the grid with a piece of filter paper. 15 µl of uranyl acetate (2%) was pipetted onto the parafilm and the grid was placed on top of it. Again the remaining liquid was wicked away with filter paper and the grid was left in the air for at least five minutes to dry completely.

3.2.3 Ultraviolet disinfection

For the ultraviolet (UV) disinfection, a 30 Watt germicidal UV lamp (model G30T8, 2.7± 0.3 mW/cm^2 irradiance at 253.7 nm wavelength, Sankyo Denki, Tokyo, Japan) was employed. The intensity was measured with a method by Rahn 1997. Two MS2 stock samples of 10^{10} PFU/ml were prepared in Eppendorf microcentrifuge tubes (up to 1 ml) at pH 3 and pH 7 in PBS. The tubes were opened and placed under the UV lamp at the same position at a distance of about 5 cm. At indicated times, the lamp was shut off in order to remove 10 µl of the sample for plating. Dilutions for plating were prepared in PBS at pH 7.5.

3.2.4 Peracetic acid disinfection

Experiments with initial virus concentrations of 10^{10} PFU/ml were performed in 25 ml glass beakers. Peracetic acid (PAA) was spiked into the buffer solution (10 ml) and the pH was adjusted if necessary with HCl (1 M) and NaOH (1 M). Then 10 µl of virus stock was added. Quenching was performed by preparing the Eppendorf microcentrifuge tubes of the first dilution step with 90 µl sodium thiosulfate (350 mg/l) before the experiment.

Experiments with initial virus concentrations of 10^{11} PFU/ml were performed in 1 ml Eppendorf microcentrifuge tubes. In these experiments, 990 µl of the 10 ml PAA solution (pH adjusted) were placed in the Eppendorf tubes. 10 µl virus stock was mixed with 10 µl of a sodium chloride solution at the appropriate pH. This was conducted to introduce aggregation before adding the virus to the disinfectant solution. The virus was then added to the PAA solution.

Before and after every experiment, the PAA concentration was measured with the peracetic acid test strips (colorimetric with test strips 5-50 mg/L, Merckoquant). The pH was also measured after the experiments, to determine any major pH change in the unbuffered solutions.

Dilutions were prepared in PBS at pH 7.5.

4 Results

In the first section, the virus was exposed to various chemical conditions as described in the introduction (2.1) to induce aggregation. In a second step the aggregated virus was disinfected and the observed kinetics was compared to the disinfection kinetics of a dispersed sample.

4.1 Aggregation experiments

The first experiment with DLS aimed to guarantee that the virus stocks in the PBS virus dilution buffer (0.5 mM PO_4^{2-}, 10 mM NaCl) were dispersed. The obtained DLS results established that the virus in the stock solution was, in fact, dispersed (Figure 2). The particle peak at 30 nm represents the virus (diameter of 29 nm). Some larger particles are always present in the solution. These larger particles can be aggregates of virus or impurities from the virus preparation or sample handling (for example PEG or dust). As this is an intensity distribution plot and not a volume distribution plot (DLS theory in the appendix), one can assume that the number of larger particles is very small in comparison to the virus peak.

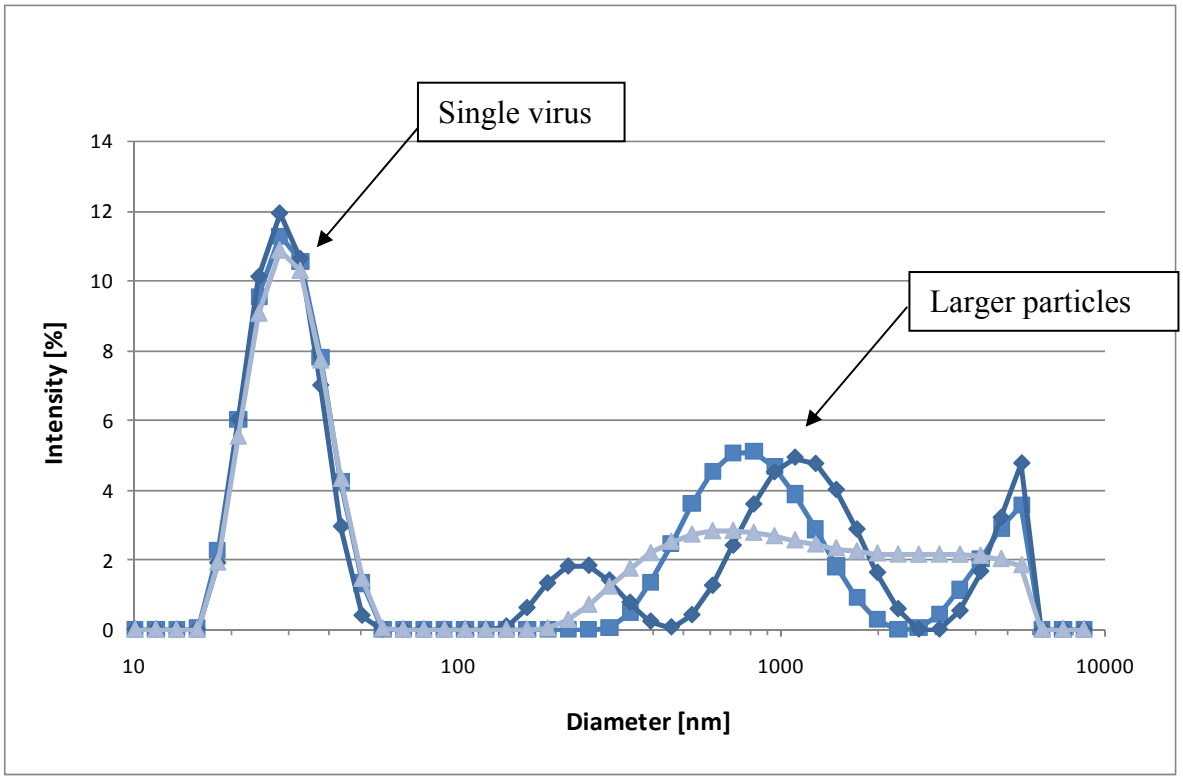


Figure 2: Intensity plot of triplicate measurements of MS2 ($5*10^{11}$ PFU/ml) in PBS (0.5 mM PO_4^{2-}, 10 mM NaCl)

In the volume distribution plot (Figure 3) the peak of larger particle is no longer observable. It is therefore clear that there are much fewer big particles in the sample than single viruses.

Brief estimations from Rayleigh scattering suggest that there are at least 10^6 times more small particles than large particles, so plating results would not be influenced.

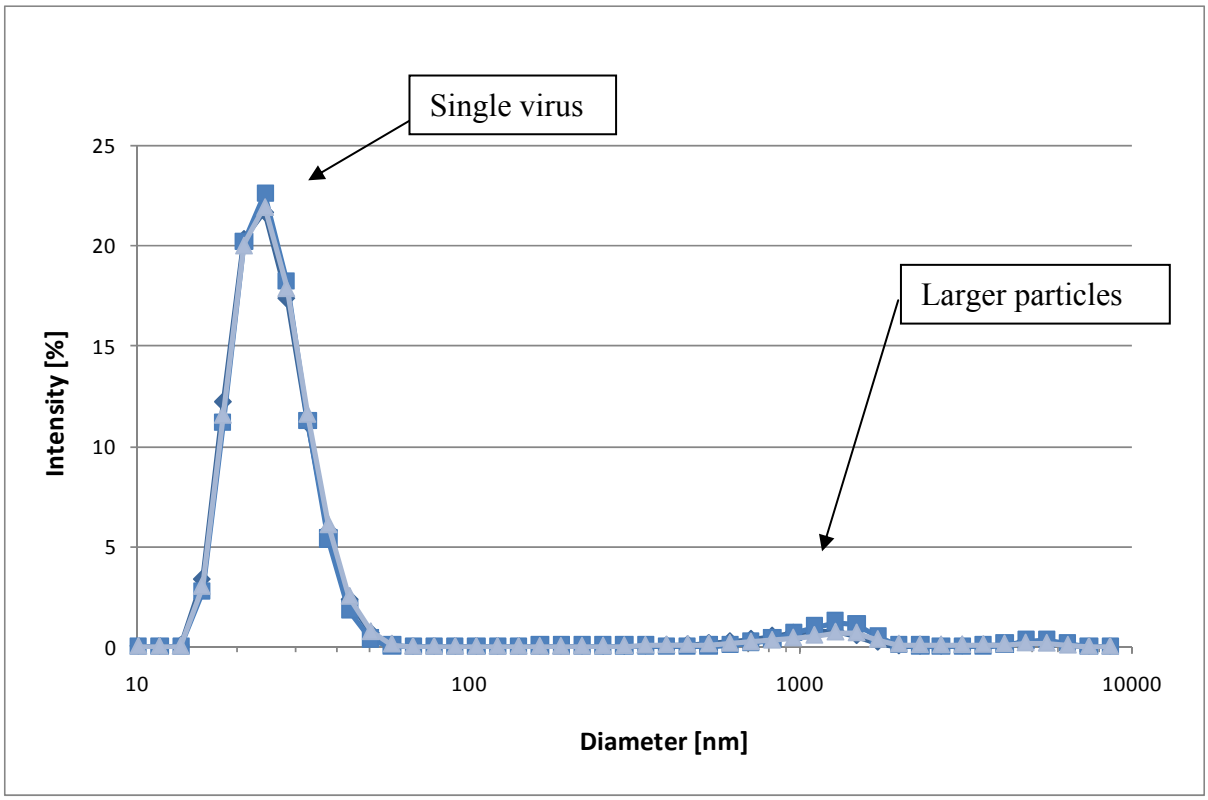


Figure 3: Volume plot of triplicate measurements of MS2 ($5*10^{11}$ PFU/ml) in PBS (0.5 mM PO_4^{2-}, 10 mM NaCl)

MS2 diluted in NaCl (20 mM), acetic acid (20 mM), and carbonate buffer (1 mM CO_3^{2-}, 10 mM NaCl) showed the same characteristic peaks with DLS analysis as with PBS. TEM-images confirmed that most of the viruses in PBS were present as single particles (Figure 4).

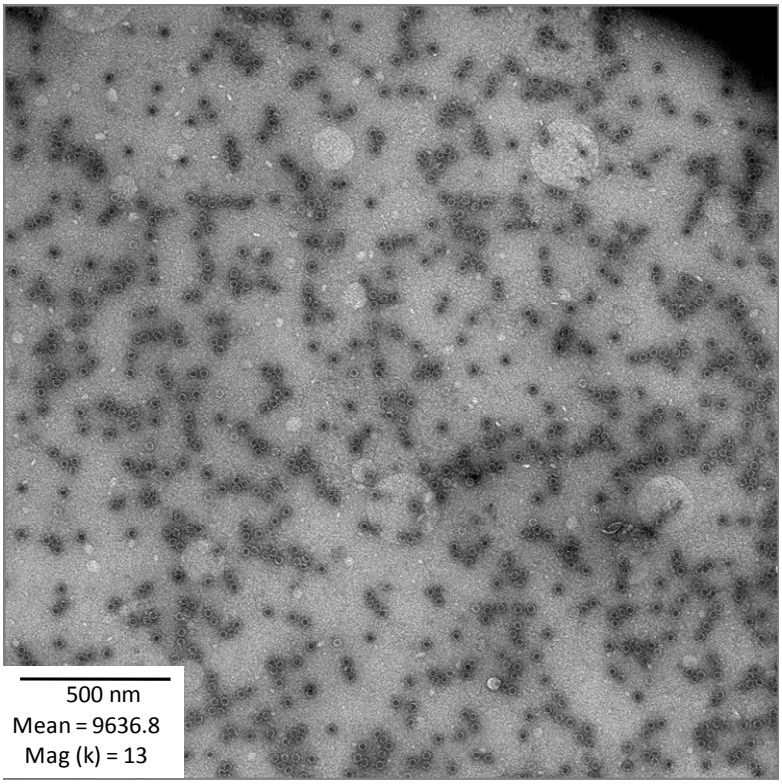


Figure 4: TEM Image of MS2 (10^{12}PFU/ml) in PBS at pH 7.5 stained with uranyl acetate

4.1.1 Ionic strength

Ionic strength showed only a minor influence on the aggregation state of MS2. To minimize effects from direct interactions of ions with MS2, only NaCl was used and thus the solution was not buffered. All experiments were performed at the pH that resulted from an unbuffered NaCl solution (~pH 6).

Only minor shifts were visible in the DLS intensity distribution plots as more salt was added (Figure 4). Intensity of the single virus peak did increase slightly and the intensity of larger particles decreased slightly with higher salt concentrations. This indicates a shift to single particles and is contrary to the double layer theory (2.1). In colloidal theory, the attraction between the particles would be greater with increased NaCl because the double layer decreases with increasing ionic strength.

In the number distributions, (not shown) no differences were observed with increasing NaCl and thus salt concentration will not have an influence on plating experiments (~10^6 times more small particles). Further experiments should be conducted to confirm this prediction.

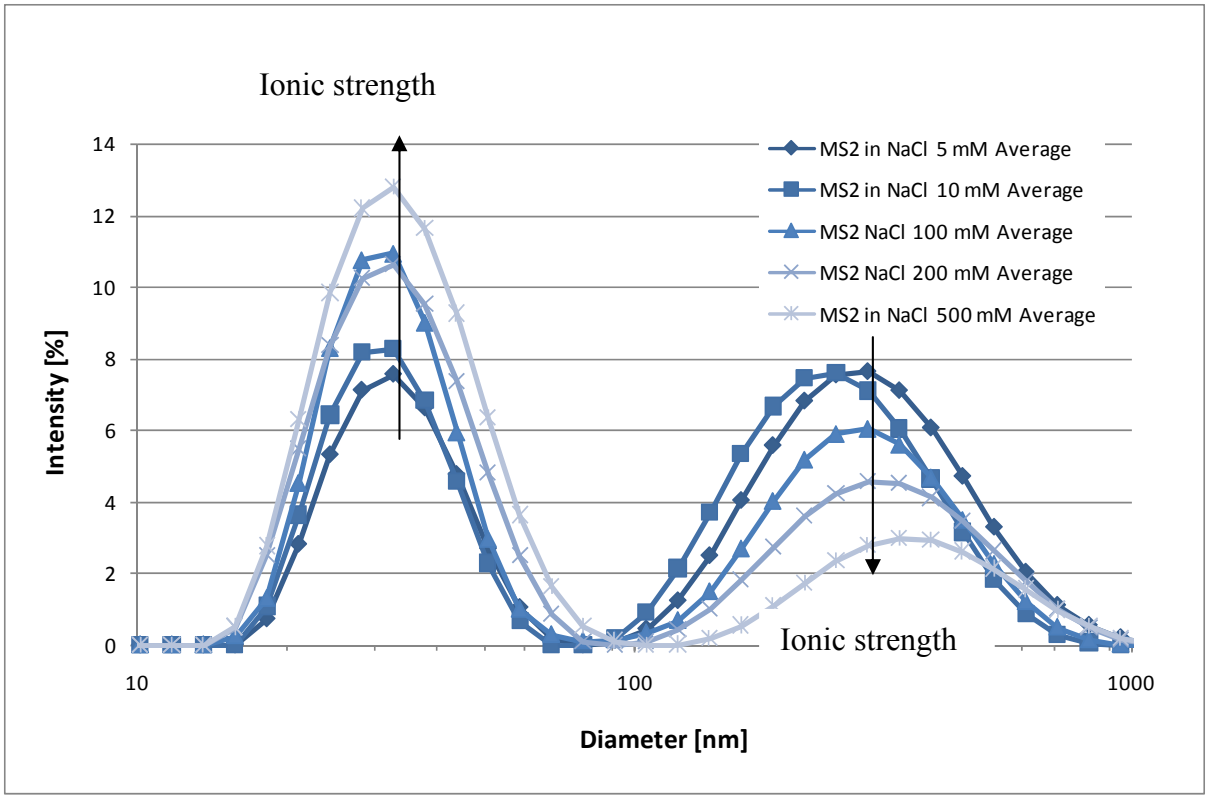


Figure 5: Size distribution of MS2 ($5*10^{11}$ PFU/ml) at different Ionic Strengths (Average of three measurements)

Floyd and Sharp (1977) used TEM to analyze samples of poliovirus with varying salt concentrations. They also confirmed dispersion in high salt concentrations and observed aggregation when poliovirus was diluted into water with concentrations lower than 10 mM phosphate or 60 mM NaCl. This is also against the colloidal zeta potential theory because high salt concentration should increase aggregation effect and not disperse virus (Equation 3). An explanation for these results could be the presence of high levels of ions binding to the virus and thus resulting in positively charged particles with an extensive double layer, which disperse with repulsive forces (Floyd and Sharp 1978a).

4.1.2 Calcium chloride

According to the zeta potential theory as discussed in the introduction (2.1), $CaCl_2$ is the most appropriate ion to induce aggregation and should be more effective than NaCl. Furthermore work conducted in other laboratories showed that the addition of $CaCl_2$ to a virus solution resulted in a decrease of PFU (Correspondence with Ofelia Romero).

Plating experiments performed with $CaCl_2$ in this study did not lead to conclusive evidence that MS2 was aggregating over time. Occasionally a slight decrease in PFU was observed in

$CaCl_2$ experiments however it was not clear whether it was produced by inactivation or aggregation. DLS and TEM were thus used to distinguish between the phenomena.

Virus samples with $CaCl_2$ analyzed by DLS did not suggest the presence of aggregates. The peak of dispersed particles is still present in the size distribution plots even at $CaCl_2$ concentrations as high as 100 mM (Figure 6). These results were similar for virus in both carbonate and Tris buffer (20 mM). PBS was not employed in the $CaCl_2$ experiments due to the possibility of forming precipitates of calcium phosphate. Even small aggregates should have a significant influence on the observed size distribution, because they scatter much more than the dispersed particles. The peak of dispersed viruses would therefore disappear completely in the presence of small aggregates, which was not the case in all experiments with DLS and $CaCl_2$.

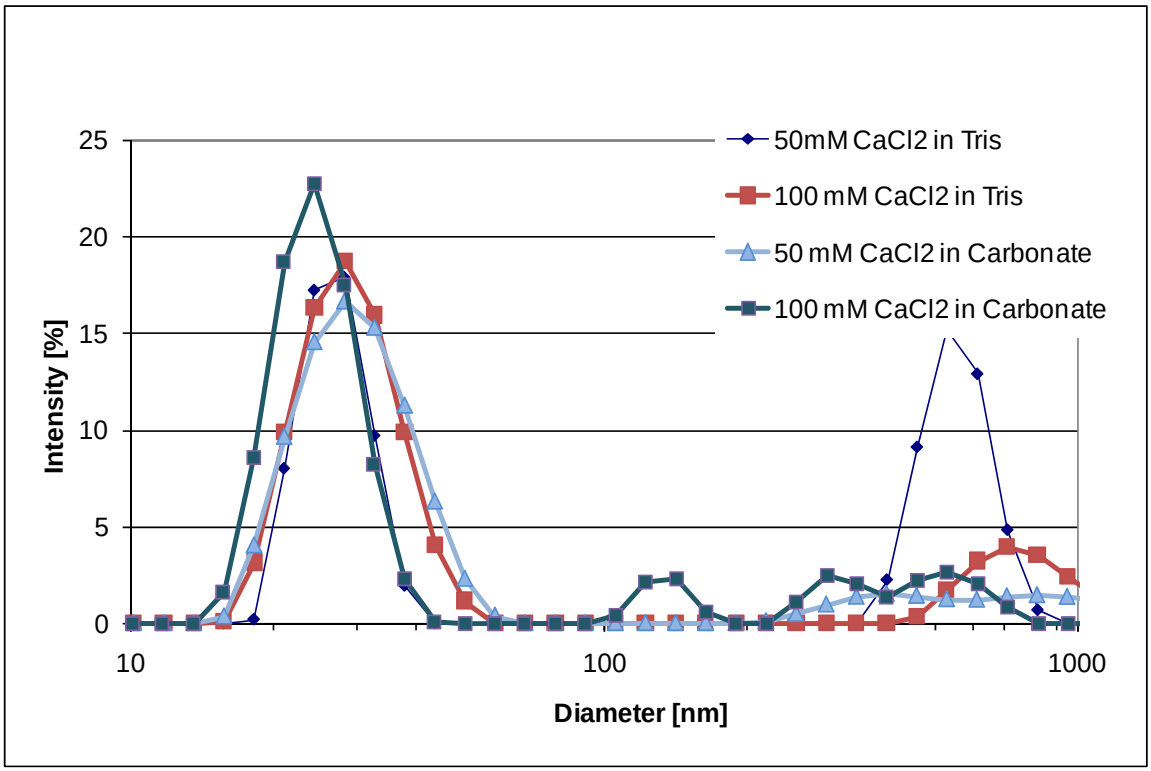


Figure 6: MS2 ($5*10^{11}$ PFU/ml) in $CaCl_2$ and Tris (20mM) or Carbonate Buffer (1 mM CO_3^{2-}, 10 mM NaCl) after shaking for 2 hours

In order to verify the DLS data, three samples were analyzed with TEM. All three samples were stirred in 1 ml Eppendorf centrifuge tubes at 5 rpm overnight (20 hours). The control in PBS without $CaCl_2$ (Figure 4, section 4.1) seems to be more aggregated than the samples with 4 mM (Figure 7) and 10 mM $CaCl_2$ (Figure 8). A possible cause of this dispersion is the increase in ionic strength, as seen with NaCl (4.1.1). These results suggest that no major aggregation is occurring due to $CaCl_2$.

It should be noted that the interpretation of the TEM images should be done with caution as the TEM preparation may affect the aggregation state. Uranyl acetate is used to stain the viruses and this can cause aggregation as the solution has a pH of 4. Additionally, when drying the sample, water is not evaporated at equal rates across the surface of the sample grid and this may also affect the aggregation state of the viruses observed with TEM.

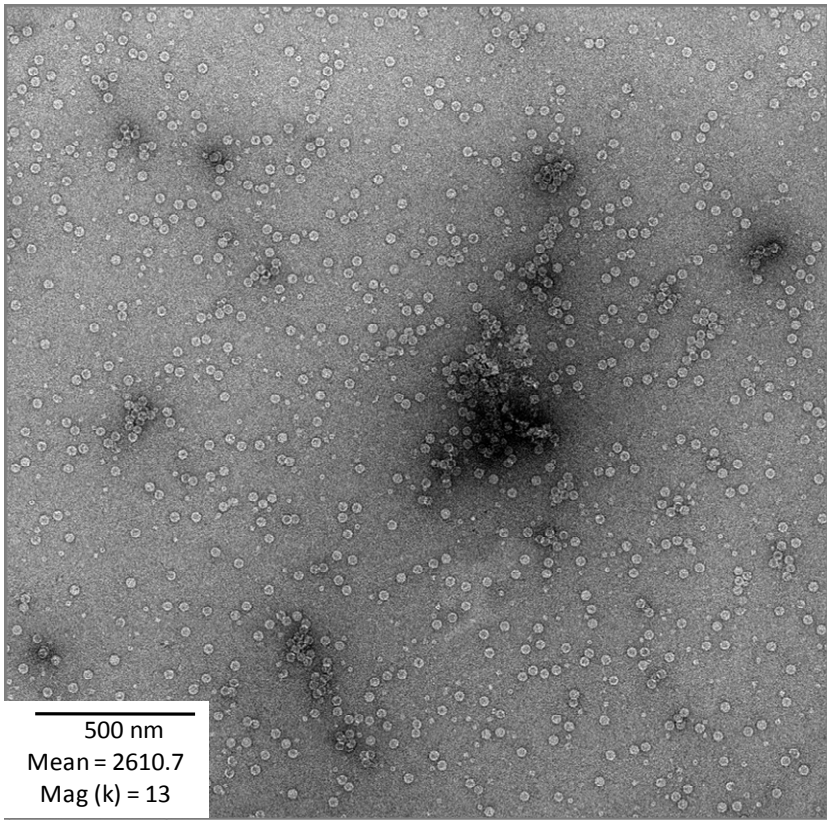


Figure 7: TEM Image of MS2 (10^{12}PFU/ml) with *4mM* $CaCl_2$ in Tris Buffer at pH 7.5 stained with uranyl acetate.

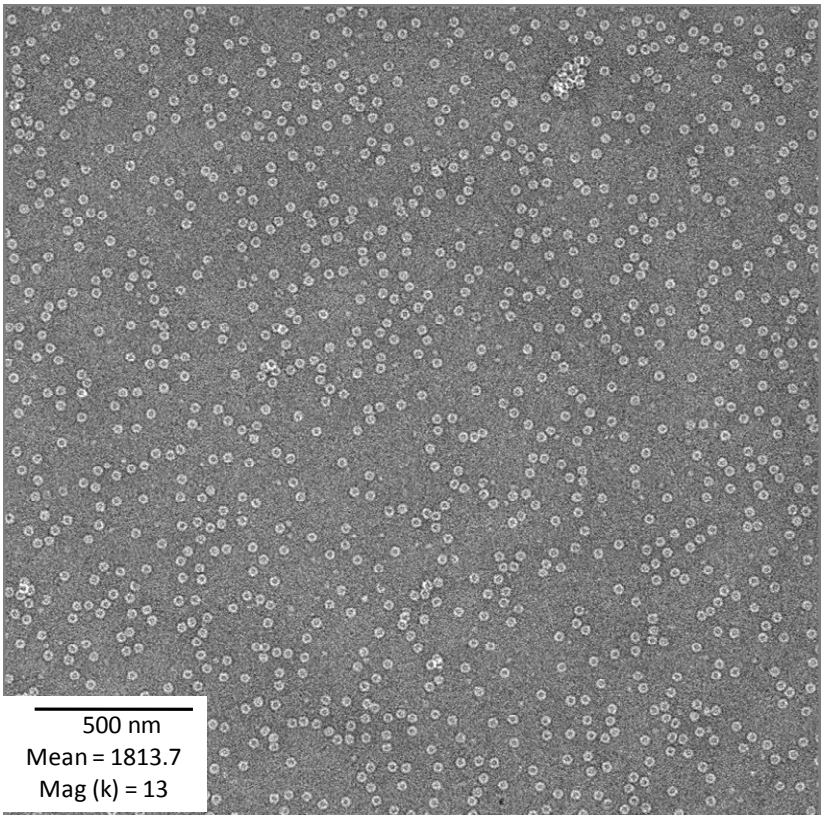


Figure 8: TEM Image of MS2 (10^{12}PFU/ml) with *10mM $CaCl_2$* in Tris Buffer at pH 7.5 stained with uranyl acetate

Contrary to these results, Floyd and Sharp (1977) did observe aggregation of Poliovirus 1 in 10 mM $CaCl_2$ at pH 7.2 (although the virus stayed mono- disperse in 1 mM $CaCl_2$). The differences in the results obtained by Floyd and the results presented here may be due to the high isoelectric point of Poliovirus of 8.2 (Floyd and Sharp 1978a) compared to MS2. Near the isoelectric point, ions can more effectively shield charge, because the overall charge of the virus is less dense.

4.1.3 pH

At neutral pH MS2 is strongly negatively charged and thus repulsive forces inhibit aggregation. In the previous sections (4.1.1 and 4.1.2) we showed that shielding of charge with ions is not enough to force MS2 to aggregate. Reducing the negative charge of MS2 by lowering the pH to the pI was tested in this section. A pH value of 3 was chosen to guarantee that MS2 is no longer negatively charged, because the pI is reported at 3.1- 3.9 in literature (Redman et al. 1997, Zerda and Gerba 1984, Langlet et al. 2008b).

The peak of the dispersed particles disappeared when the pH was lowered to pH 3 in PBS (Figure 9, blue lines). After filtering the sample at pH 3 through a 100 nm filter, the correlation in the correlogram was very weak and could not be analyzed further (data not

shown). This indicates that most virus particles were eliminated by the filter and thus virus aggregates of a diameter larger than 100 nm were formed.

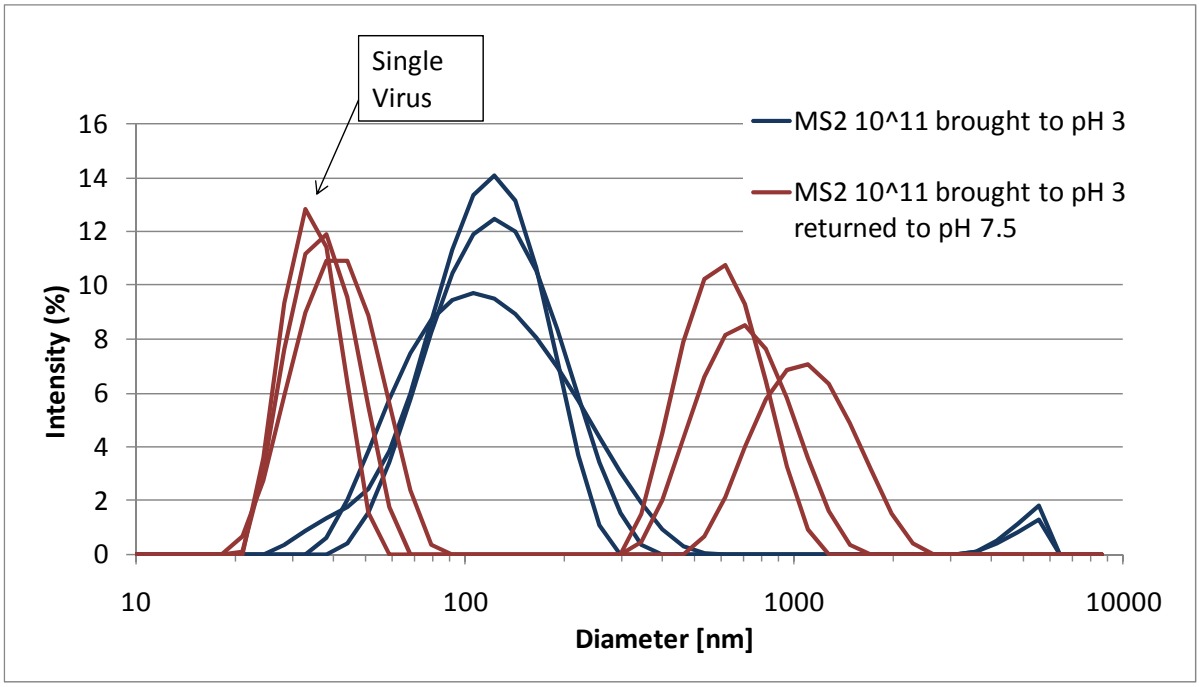


Figure 9: Triplicates of the intensity distribution of MS2 ($5*10^{11}$ PFU/ml) at pH 3 and pH 7 returned from pH 3 in PBS (5 mM PO_4^{2-}, 10 mM NaCl)

Interestingly this reaction seems to be reversible. When the sample pH is adjusted back to pH 7, the single virus peak comes back instantly, although some big particles are still present (Figure 9, red lines). Reversibility was also observed in plating data (not shown). These results are important when considering virus disinfection because they suggest that if viruses are in an aggregated state during disinfection, they may become dispersed after treatment and thus result in an increase in PFU, and reflect the number of single viruses. Floyd and Sharp (1977) also reported reversibility of aggregation with poliovirus when changing the aggregation state of the virus by returning from pH 5 (aggregated) to pH 7 (dispersed).

The rate of aggregation when lowering the pH is very fast. A large shift in the z-average size occurs in the first minutes (Figure 10). The z-average size then increases slowly. At 30 minutes no more augmentation was visible. Floyd and Sharp (1977) found comparable time for poliovirus aggregation in low pH. They obtained a levelling off of aggregation at 20 minutes with the SPA test (elimination of big particles by centrifugation).

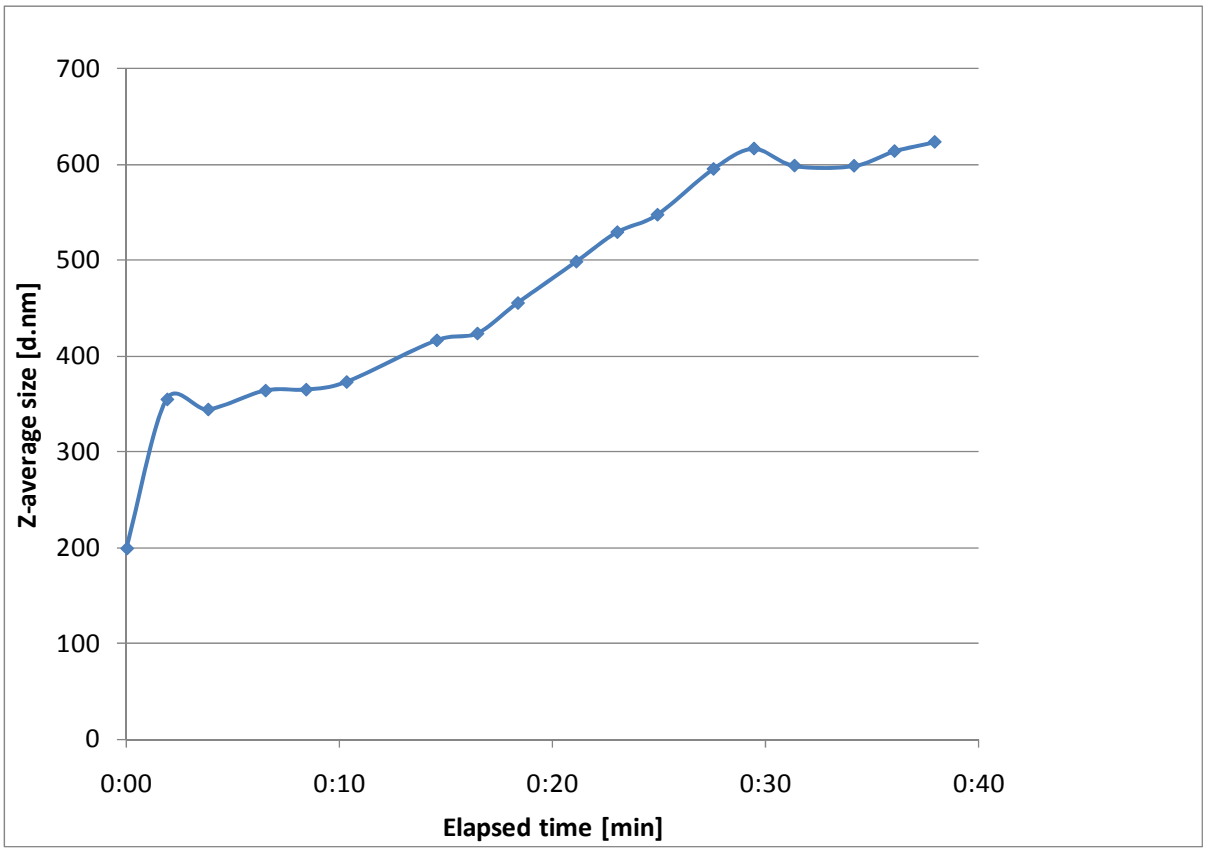


Figure 10: Shift in average size over time after lowering to pH 3

TEM images of MS2 in phosphate buffer at pH 3 confirmed aggregation at low pH (Figure 11). The viruses were mostly in aggregates containing approximately 10-20 viruses. To confirm the aggregated state was not caused by high virus concentration, a diluted sample was also analyzed (Figure 12). Even in the diluted sample viruses had the tendency to form aggregates. These results agree with analysis by DLS, the aggregates were not extremely big, but in the range of several 100 nm (Figure 9).

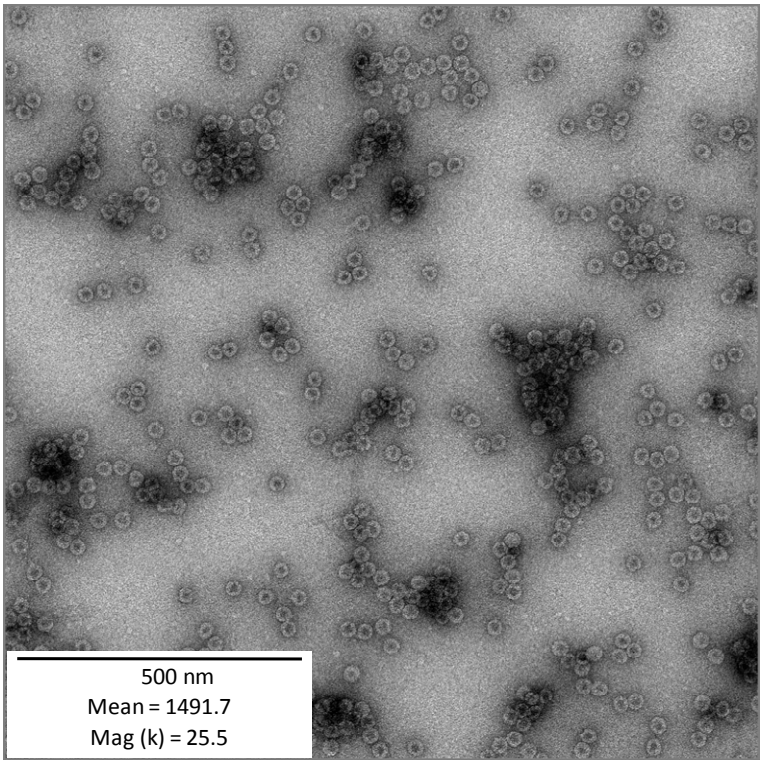


Figure 11: TEM image MS2 10^{12} PFU/ml at pH 3 stained with uranyl acetate

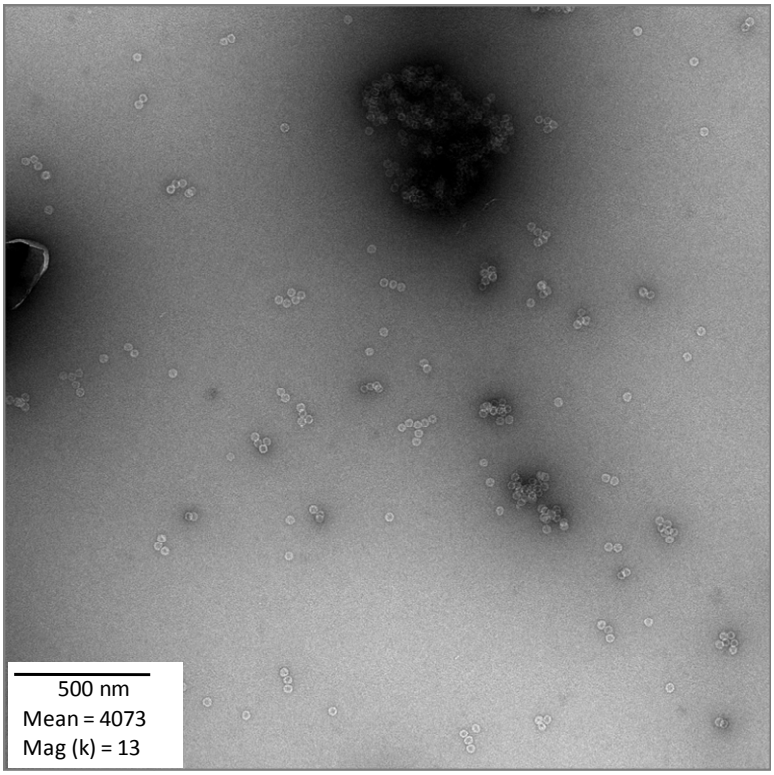


Figure 12: TEM image MS2 $3.3*10^{11}$ PFU/ml at pH 3 stained with uranyl acetate

To quantify the level of aggregation, samples were prepared in PBS at pH 3 and two dilution series were made: one at pH 3 (to count the number of aggregated virus particles) and one at

pH 7 (to disperse the virus and count the number of active virus). A loss of one log was consistently observed with plating at pH 3 (Figure 13). Plating at pH 7 reversed the aggregation and no loss was observed. The one-log loss observed in pH 3 should lead to aggregates of approximately 10 virus particles. This result cannot be used to quantify exactly the amount of aggregation because of two major problems: (i) Plating results were sometimes not countable; i.e. when the dilution series were conducted at pH 3 to obtain aggregation states, a trend was observed where one dilution point had too many PFU to count and the next dilution point contained zero PFU (ii) it was not possible to plate at pH 3 because the last step of plating involves dilution in soft agar with a higher pH. Problem (i) was due to adsorption of virus to the Eppendorf tube walls. The virus particles are near the isoelectric point and are therefore not charged. The uncharged particles can thus adsorb to the uncharged walls of the Eppendorf centrifuge tube. Therefore low concentrations of virus at pH 3 will always adsorb to the wall and dilution series at pH 3 are therefore not possible. For problem (ii) the plating procedures must be adapted to pH 3.

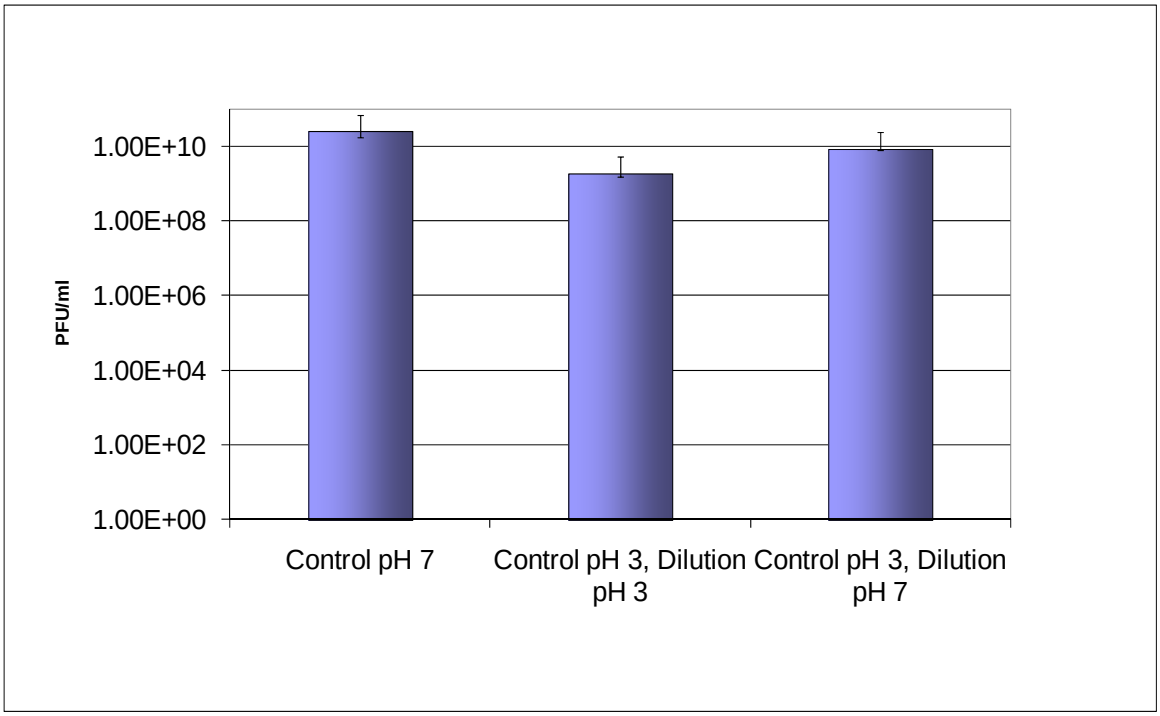


Figure 13: Plating of aggregated MS2 (10^{11} PFU/ml) with dilution in PBS (5 mM PO_4^{2-}, 10 mM NaCl) at pH 3 and pH 7 (error bars represent the 95 % confidence interval from triplicate measurements)

The adsorption phenomenon of virus on plastic bottles is described by Butot et al. (2007) who saw immediately attachment of hepatitis A virus to bottles and suggested that it was due to the proximity of its pI to the water pH.

Another characterization of the aggregation size was conducted by the sequential filtering of the aggregated sample through different filter pore sizes (0.45 µm, 0.22 µm and 0.1 µm, Durapore®Membrane Filters, Millipore) then plating the filtrate at pH 7 (to disperse the aggregates). A control sample at pH 7 was also filtered in order to make sure that dispersed virus was not lost during the filtration step. No losses in PFU were observed in the control samples after filtration (data not shown).

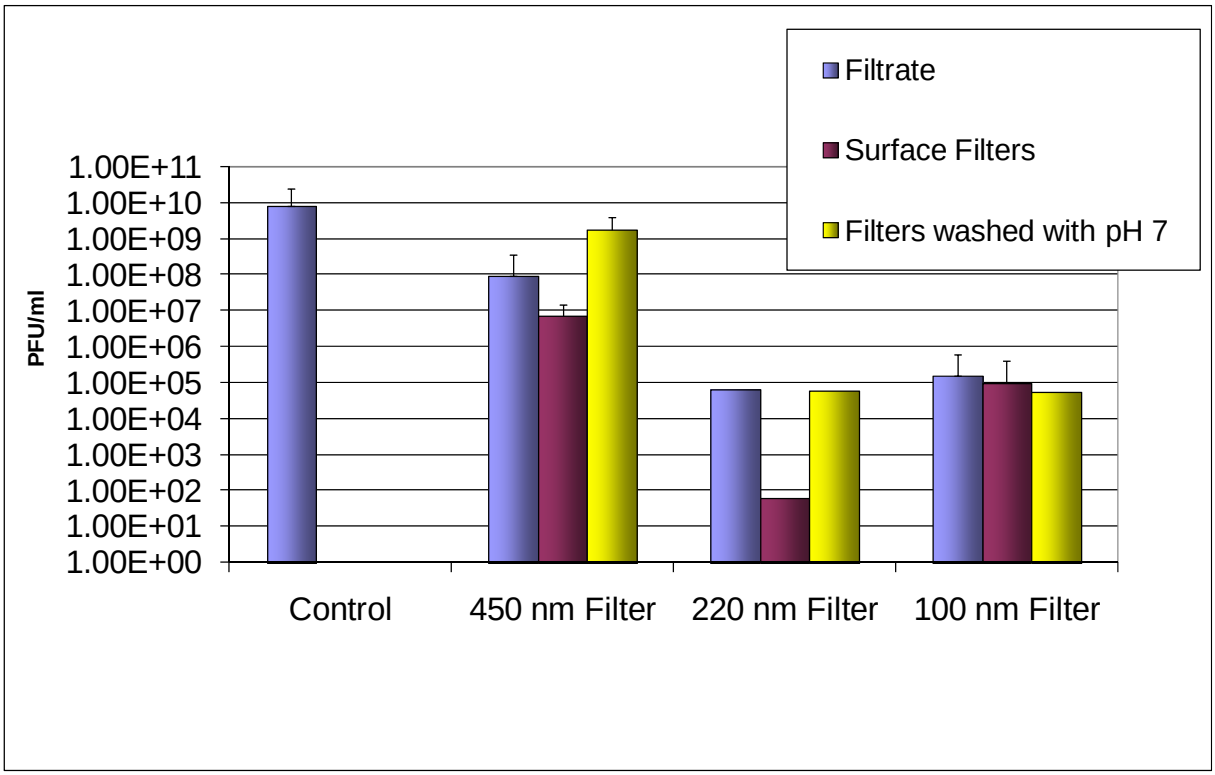


Figure 14: Filter experiments with MS2 10^{10}PFU/ml in PBS (5 mM PO_4^{2-}, 10 mM NaCl) at pH 3 (error bars represent the 95 % confidence interval from triplicate measurements)

About two logs of PFU were lost in the first filter step with 450 nm pores (Figure 14, filtrate). Assuming that we have negligible adsorption to the wall and the membrane filter, two logs of viruses were larger than 450 nm in diameter The next filtration step (220 nm) shows a decrease of 3 logs, while the last step (100 nm) shows no decrease at all. No loss of PFU at the last step was surprising because the sample was treated like before and should consequently show adsorption occurring as well. These results suggests a distribution of the aggregate diameters as the following: 99 % over 450 nm, ~1 % between 220- 450 nm and only 0.0001 % below 100 nm. Therefore 5 logs of virus would be in an aggregated state. This disagrees with the TEM images (Figure 11, Figure 12) and DLS data, which only show small aggregates of 10- 20 viruses with a diameter at 100 nm. It was thus concluded that adsorption plays a role in the filtering process.

To further investigate the effect of adsorption, the filter surface was washed with PBS at pH 3 after filtering the virus (Figure 14, surface) so aggregates on the surface could be collected and quantified. After this treatment the filter was washed with PBS at pH 7 to recover adsorbed virus particles. Most of the viruses were recovered with the pH 7 and not with the pH 3 surface wash (Figure 14). Therefore it was concluded that adsorption plays a major role at pH 3 and a realistic estimation of the size of the aggregates by filtering was not possible.

Further studies have to be conducted to quantify the amount of adsorption. For the work reported here, disinfection experiments were performed with sufficiently high concentrations of virus so that adsorption only played a minor role. Dilution series were always prepared in PBS at pH 7.5 to avoid adsorption of virus to the Eppendorf centrifuge tubes used in the dilutions and to disperse aggregated virus. At pH 7.5, no adsorption was observed.

Measurement of the electrophoretic mobility in PBS did not show a pI in the range of 3.1-3.9 as given in literature (Figure 15). The electrophoretic mobility experiments gave similar results for both NaCl (20 mM) and PBS. For this experiment, MS2 (10^{13} PFU/ml) was diluted 100 fold in 20 mM NaCl without a buffer exchange (measurements with buffer exchange are explained later). The absence of the pI may be due to the binding of phosphate to lysine on the MS2 surface, as suggested by Yuan et al. (2006). Dilution into NaCl (20 mM) could not have sufficiently remove phosphate binding to the virus surface.

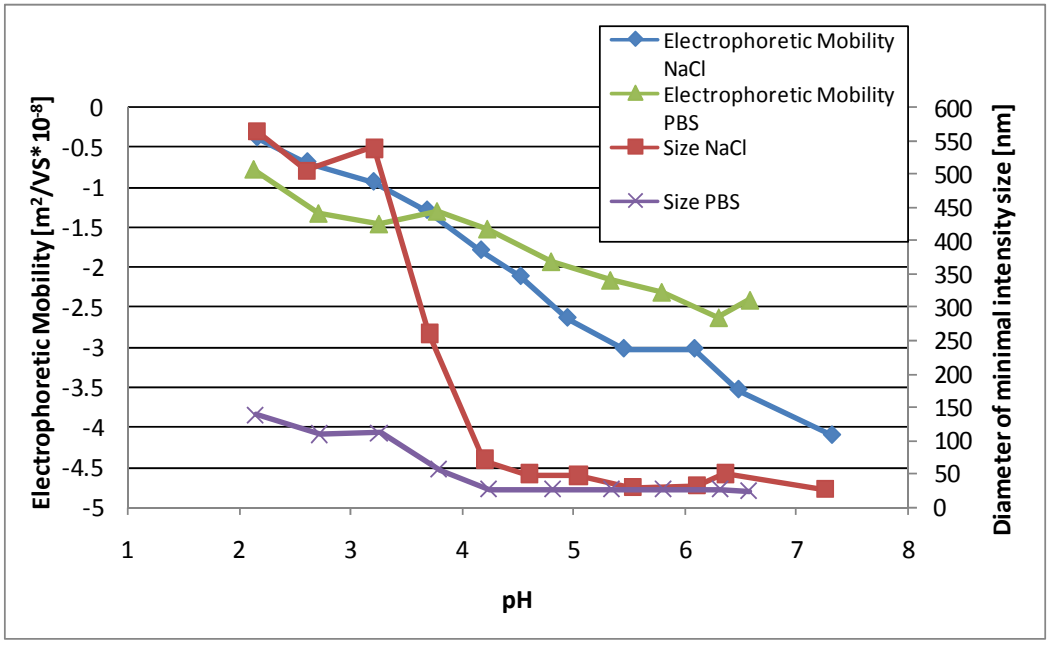


Figure 15: Electrophoretic mobility and minimal size detected in volume distribution of MS2 as a function of the pH: MS2 10^{12} PFU/ml for PBS and $2*10^{11}$ PFU/ml for NaCl (20 mM)

Figure 15 also shows that the minimum observed size began to increase at pH 4, which is near the isoelectric point in literature. However this point was not measured by the electrophoretic mobility. Below pH 4 the size increased much more with 20 mM NaCl than with PBS.

Size comparison of MS2 in PBS, acetic acid (20 mM) and NaCl (20 mM) at pH 3 (Figure 16) confirmed the results seen in titration experiments. In PBS at pH 3, MS2 formed aggregates of 100-200 nm, while in 20 mM NaCl and acetic acid aggregates seem to be at ~1 µm.

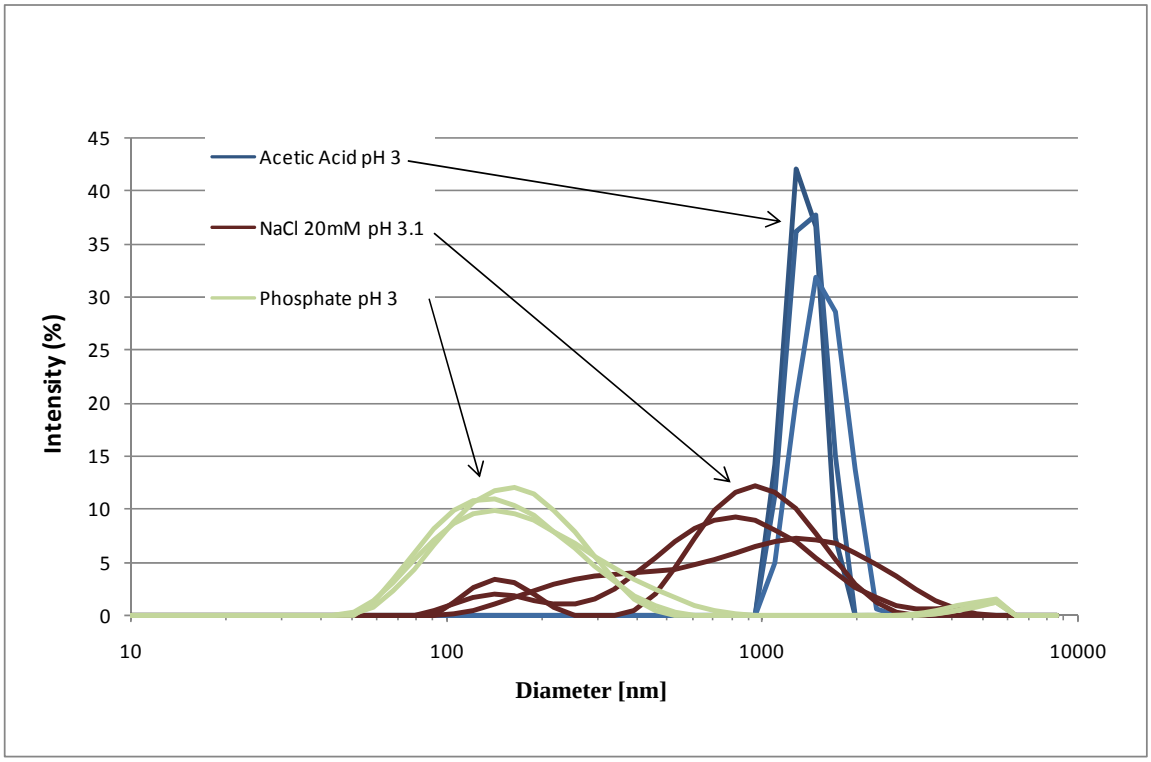


Figure 16: MS2 (10^{11} PFU/ml, except NaCl $5*10^{11}$PFU/ml) in pH 3 with different Buffers

Electrophoretic mobility was then measured on virus samples in 10 mM NaCl after performing a buffer exchange to rid the solution of phosphate (Figure 17). 10 mM NaCl was chosen because high amounts of NaCl may cause dispersion (4.1.1). The zeta potential was calculated using Ohshima's approximation for hard particles (Equation 4) and Equation 2. A $f(\kappa a)$ value of 1.2 was calculated with this approximation.

As observed in Figure 17, the zeta potential crosses the origin during the titration when the virus was in 10 mM NaCl after a buffer exchange step. This was not observed in the NaCl solution without a buffer exchange step. The pI by titration from high to low pH was observed at 3.64 and this is very close to the point in literature, given at pH values of 3.5, 3.9, 3.1 and 3.9 (Redman et al. 1997, Zerda and Gerba 1984, Langlet et al. 2008b [at 1 mM and 100 mM

$NaNO_3$], respectively). This also corresponds well with the theoretically calculated value of pH 3.8 by Schaldach et al. (2006).

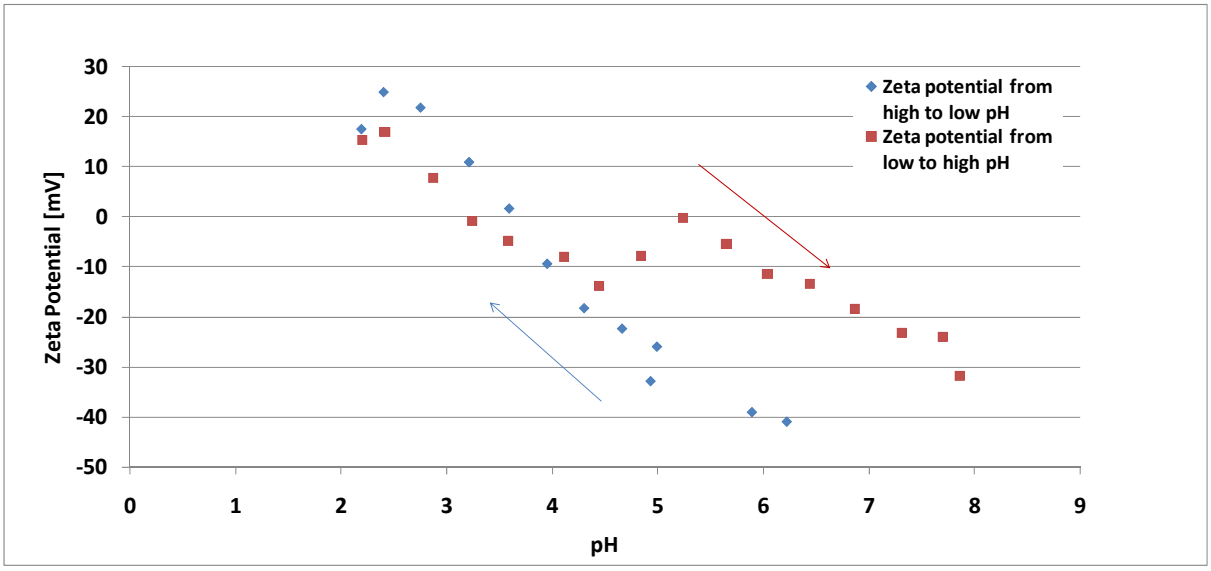


Figure 17: Zeta potential in 10 mM NaCl (MS2 10^{11} PFU/ml)

Titration with the same sample backwards indicated a pI at pH 3.2. Also a significant shift was observed at pH 4.5. The difference between the pIs can be explained by different reaction times of the system. The shift could be caused by the abrupt dispersion of aggregated MS2 and thus abrupt presence of still positively charged virus (from inside the aggregate), but more data is needed to prove this hypothesis.

The diameter was also measured by DLS during this titration and the results show an increase in the z-average size near pH 4 (Figure 18). By taking only the minimal sizes in intensity as well as in volume distribution, large particles, which can influence the average size, are eliminated. The volume distribution increases later because it has a great sensitivity for small particles (see DLS theory, appendix). At a zeta potential of ±20 mV a colloidal system normally becomes unstable and aggregates. This agrees with the employed zeta potential estimation for our samples because -20 mV is crossed at pH 4.2 (Figure 17) which is very close to the pH where aggregation is observed (Figure 18).

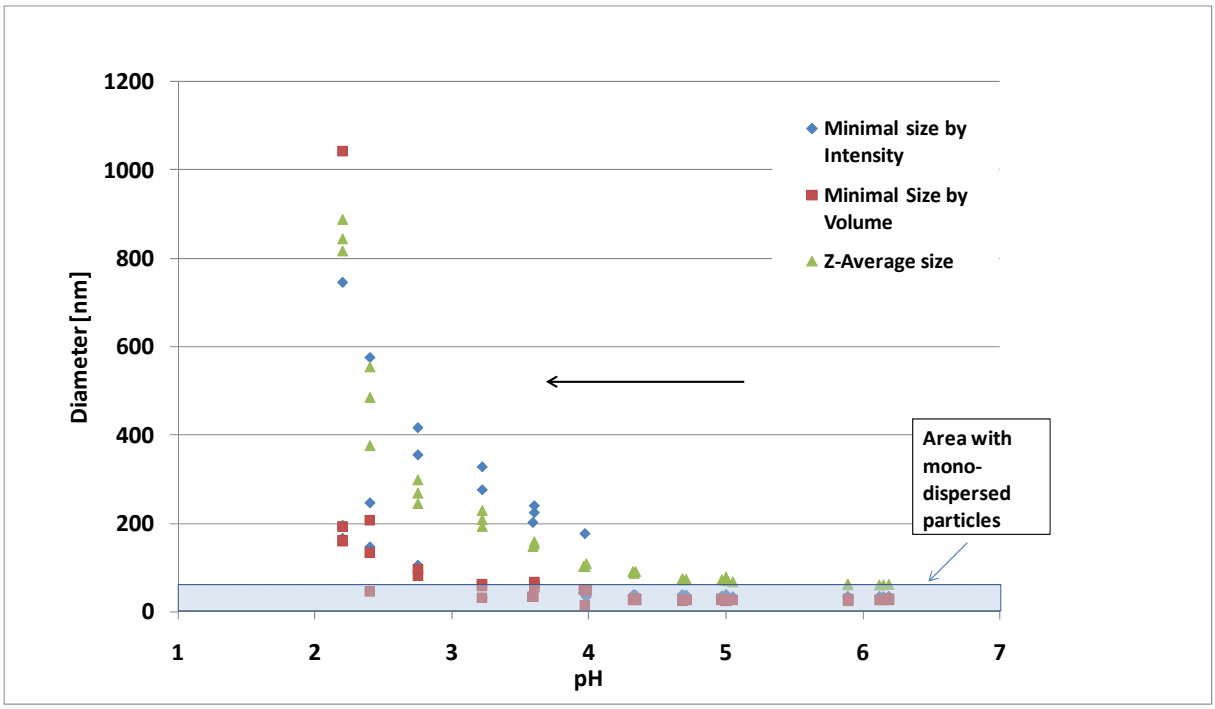


Figure 18: Size measurements of titration from high to low pH (10^{11} PFU/ml)

A reversed titration with the same sample back up to pH 8 is less defined (Figure 19). The z-average results indicate a decrease in size with increasing pH, although the intensity and volume size values varied substantially with repeated measurements.

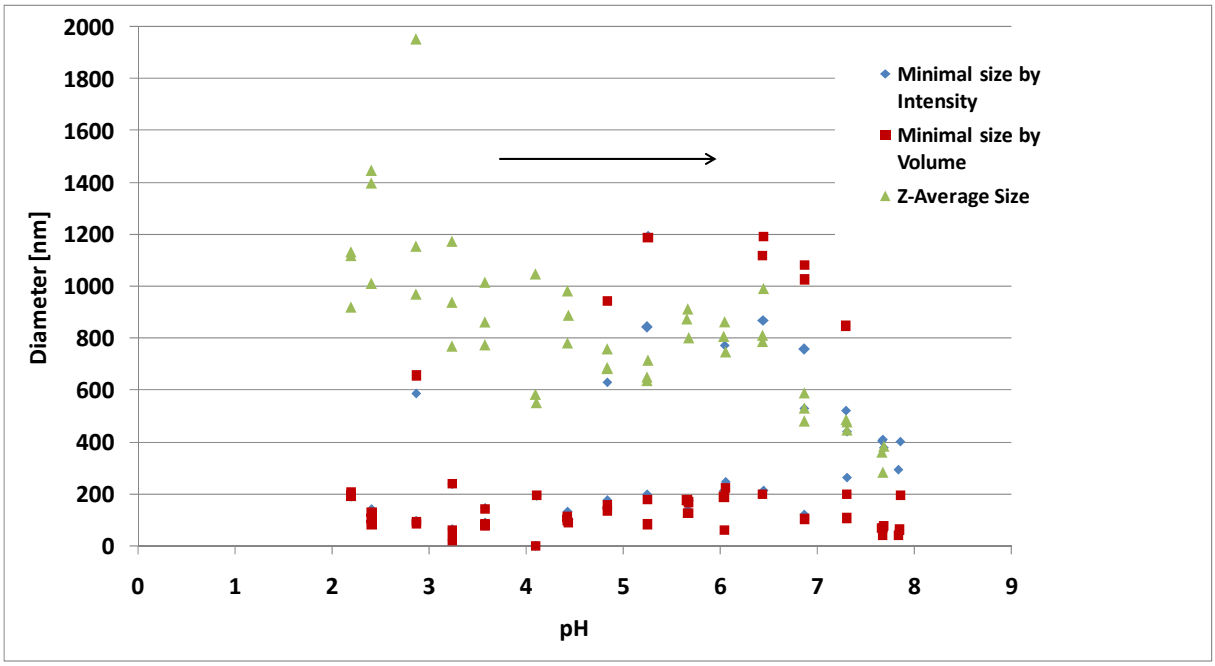


Figure 19: Size measurements of titration from low to high pH (10^{11} PFU/ml)

Possible causes are: (i) lowering to pH 2 could destroy the virus and release proteins and DNA, which can be involved in the combining of virus to form aggregates (ii) some larger settling particles can cross the laser beam and influence in these measurements, and (iii) dispersion of large aggregates takes too much time, so they are still present in the solution. This experiment will be repeated by starting with a fresh sample at pH 3 and longer time steps.

4.1.4 Inhibition of aggregation by ionic strength

This section describes the influence of salt on the aggregation at pH 3. After a buffer exchange in 20 mM NaCl, MS2 was added to Eppendorf centrifuge tubes with varied salt concentrations. The size of the virus in the sample was measured with DLS and then 10 µl of 0.025 M HCl added to the sample volume of 250 µl. The resulting pH was measured with pH paper. All samples were at pH 3 after addition of HCl.

Before the addition of HCl no aggregation was observed (data not shown). After acid addition only the samples with low ionic strength aggregated as seen by the augmentation of the z-average size in Figure 20. The turning point seems to occur around 100 mM, but further experiments will need to be conducted to establish this exact relationship. Inhibition of aggregation by high ionic strength was also seen by Floyd and Sharp (1977). In their study pH induced aggregation at pH 6 with poliovirus and aggregation was inhibited with 100 mM NaCl or 10 mM magnesium. The concentration needed to prevent aggregation was pH dependent. In further studies, they established that mono- and divalent ions can enhance aggregation but also prevent aggregation at higher concentration (Floyd and Sharp 1978a). Divalent ions were much more effective in preventing aggregation. Penrod et al. (1996) observed less adsorption to quartz grains at pH 5 and ionic strength from 0-300 mM for MS2 than for the virus Lambda, even if the measured electrophoretic mobility was less for MS2. They also showed an increase of attachment efficiency of MS2 from 0-100 mM ionic strength, but a decrease at higher ionic strength (>100 mM). They calculated the interaction energy based on a balance of attractive van-der-Waals and repulsive steric interactions from coat proteins and came to the conclusion that steric interactions can play an important role in adsorption. Steric interaction could also induce inhibition of aggregation like seen in Figure 20. Contrary to this study Langlet et al. (2008b) did not observe a dependency of aggregation of MS2 on the ionic strength. They tested aggregation of MS2 over a pH range from 2 to 7 at 1 mM and 100 mM $NaNO_3$ and both ionic strengths showed a reduction of the diffusion coefficient (which is related to aggregation, see appendix) at pH 4. This experiment should be repeated with divalent ions and with varied buffer concentration and ionic strength in order to

verify the influence on aggregation. Also longer time scales should be tested because longer exposure could lead to aggregation even with high ionic strength.

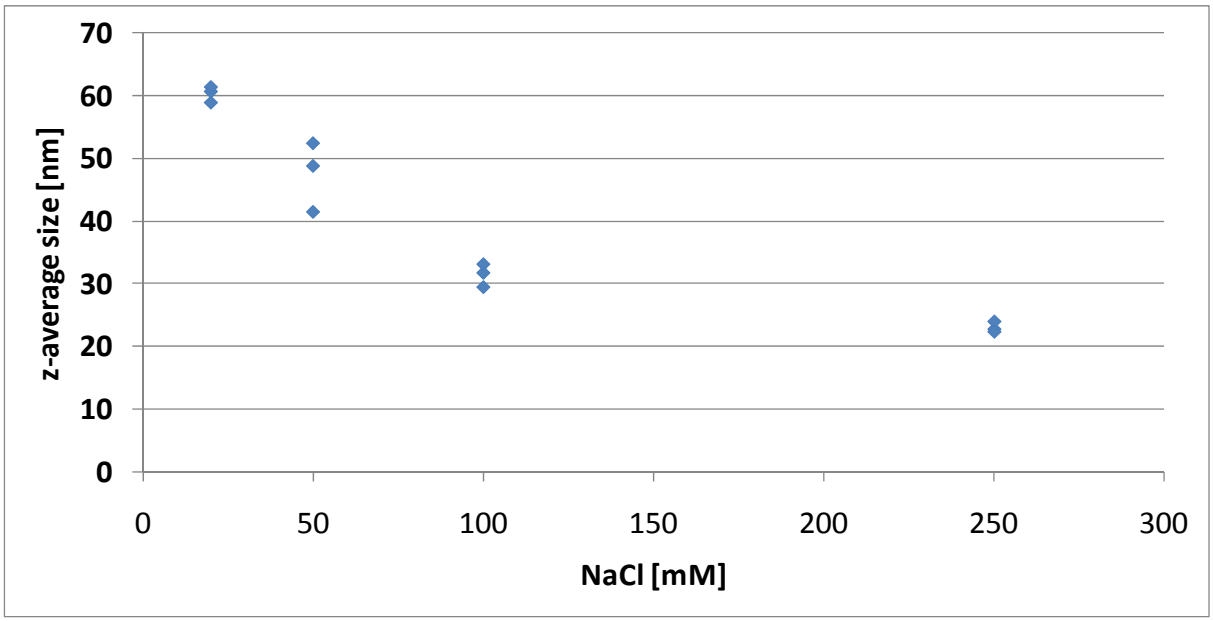


Figure 20: Inhibition of aggregation by salt concentration (MS2 10^{11}PFU/ml)

Addition of salt to an aggregated solution at pH 3 did not result in dispersion. Further investigation concerning this observation should be performed.

4.2 Disinfection

4.2.1 UV

UV disinfection is very efficient for a solution of viruses without high levels of other materials (Hijnen et al. 2005). In this study a 4 log reduction was observed with 2.7± 0.3 mW/cm^2 irradiance at 253.7 nm wavelength after 80 seconds for a solution at pH 7 (Figure 21). At pH 7, the reduction rate (k= 0.0195) was much slower than at pH 3 (k= 0.036), thus a greater inactivation for the aggregated solution. The observed reduction rate at neutral pH is much lower than described in literature. Hijnen et al. (2005) compared the disinfection kinetics of five studies with MS2 at monochromatic light and solution in effluent or tap water and calculated a mean k-value of 0.055, which is more than twice as high as the k-value seen in this study. A more similar inactivation rate (k= 0.0389) was observed by Simonet and Gantzer (2006) at pH 7.2, although the phosphate buffer concentration was not specified in the paper.

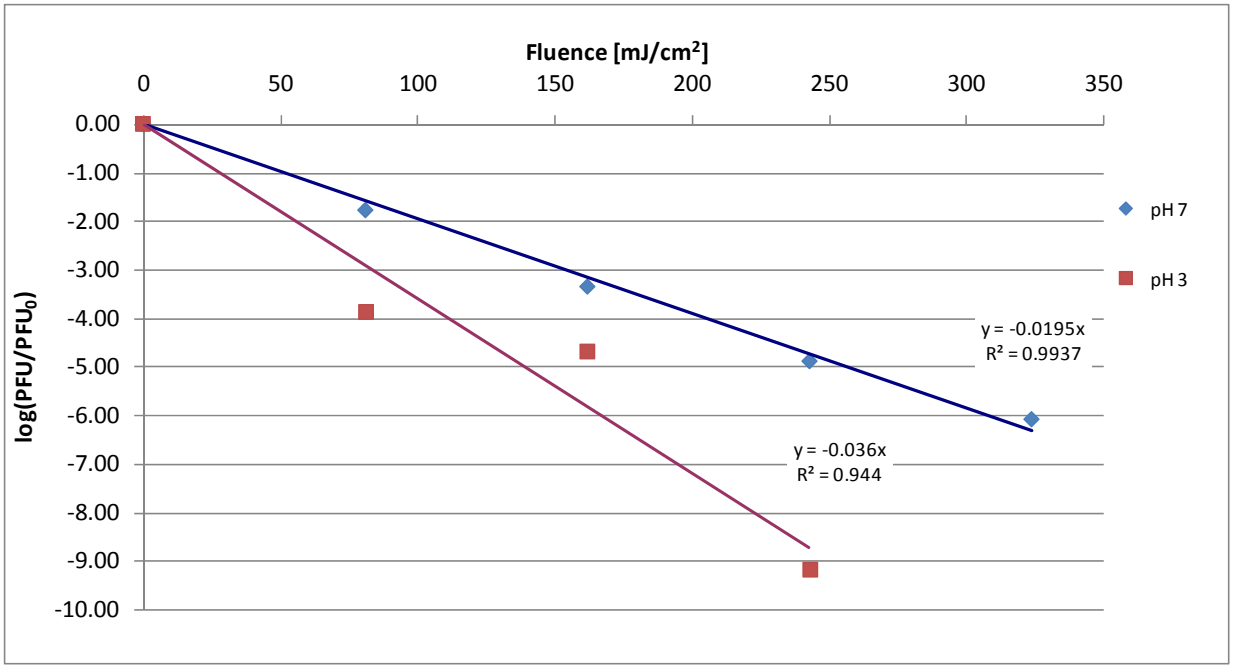


Figure 21: UV-Disinfection of MS2, initial MS2 concentration 10^{11} PFU/ml

Galasso and Sharp (1965) compared the UV disinfection rates for dispersed and aggregated vaccinia virus. Slower inactivation was observed with increased aggregation and they attributed this to the fact that aggregates, like organic matter, can shield viruses from irradiation. Unlike the experiments conducted in this study, where aggregation state was controlled by pH, they resuspended aggregates with rigorous pipetting.

An explanation for the higher inactivation rate observed at pH 3 in this study could be that most amino acids of the coat protein of MS2 have a pI of 5-6 and are thus protonated at pH 3. Direct oxidation (2.2) of the amino acids could thus be influenced by the pH, because the electron structure and thus the adsorption spectra of amino acids changes with protonation. Indirect oxidation of amino acids by ROS is pH dependant (for example singlet oxygen and histidine, Bisby 1999) and can thus change the disinfection efficiency. Furthermore, production of ROS by proteins (Davies 2003) could be dependent on the pH, but little research has been done on this subject.

Davies-Collie et al. 1999 observed a pH dependency of UV disinfection with *E.coli*. Inactivation rates of *E.coli* with singlet oxygen are greater at pH 9.5 than at pH 7.5, but most authors have not observed a pH dependency in the pH ranges examined in this study (Kohn and Nelson 2007, Curtis et al. 1992).

UV-disinfection was not further investigated, because other effects caused by the pH may play a more important role in UV disinfection rates than the virus aggregation state.

4.2.2 Peracetic acid (PAA)

For disinfection experiments with PAA, pH values below and above the virus pI were chosen (pH3 and pH 5). Experiments were conducted to confirm that neither a concentration change in PAA nor a shift in the pH was observed during the time span of all experiments. Based on preliminary studies, the results suggest that the rate of disinfection with peracetic acid has no concentration dependency in the range of 5 to 20 mg/L (Figure 22). The system is thus saturated with disinfectant and with n=0 in the Chick-Watson model (equation 5), the decay rate of PFU only depends on the time. Further studies have to be conducted to confirm these results. Maillard et al. (1994) saw a 4 log reduction of MS2 at pH 1.5 with a 1% PAA solution (100 g/l) in 20 minutes. This is only two times faster than the rate observed in this study, but they employed a 500 fold higher concentration of PAA and thus confirm the observed results. Jensen et al. (1980) report a similar of level saturation for the disinfection of coxsakievirus B5 with chlorine when concentrations were higher than 30 µM.

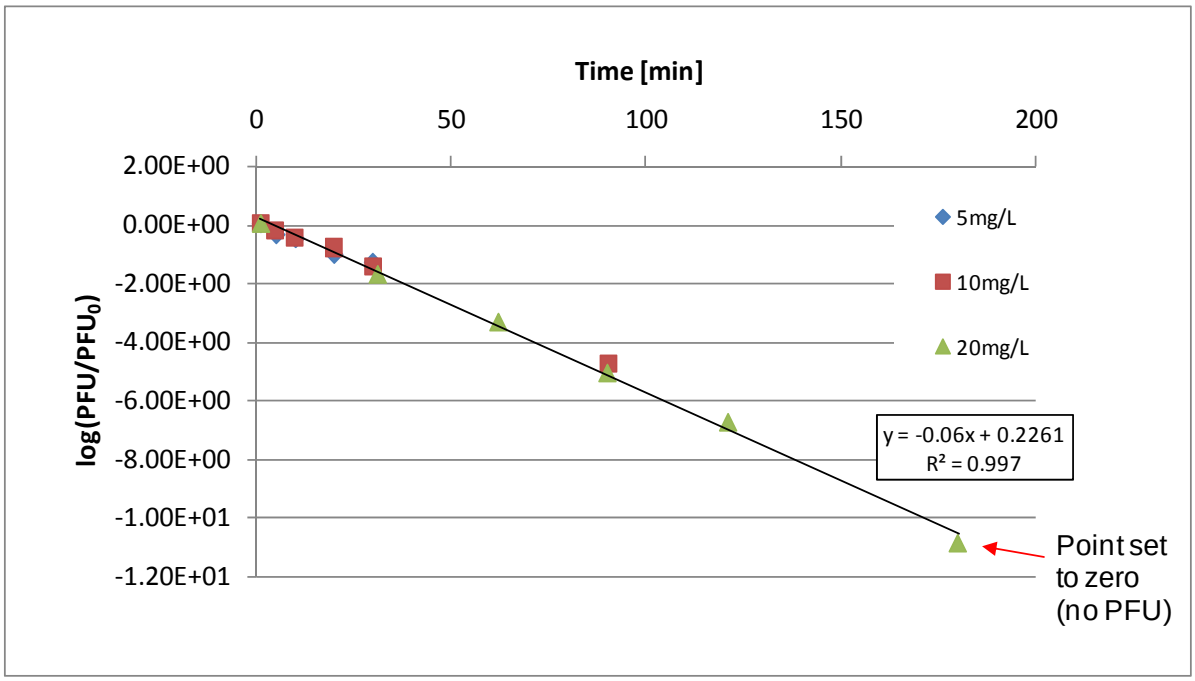


Figure 22: Disinfection times at different PAA concentration (MS2 10^{11}PFU/ml)

All experiments were performed at a peracetic concentration of 20 mg/L so as to avoid a significant loss of peracetic acid throughout the experiment. Inactivation experiments at pH 3 and 5 in PBS did not show differences in kinetics until the loss of 6 orders of magnitude (Figure 23). Afterwards PFU decrease much slower at pH 3 (tailing). This tailing of the inactivation could be due to the presence of a few big aggregates, while most viruses form smaller aggregates of less than 16 particles (2.2). The results of TEM and DLS agree well

with this assumption. DLS experiments results indicated a mean diameter around 150 nm, and TEM images showed small aggregates with less than 16 particles. Larger particles were not seen in TEM, but appear in the intensity distribution in DLS. Further studies are necessary to confirm the presence of such a particle distribution.

The effect of tailing in disinfection was also seen by Sharp et al (1974) and Floyd and Sharp (1977). They observed this tailing when Reovirus was disinfected with bromine and related it to the presence of a few large aggregates.

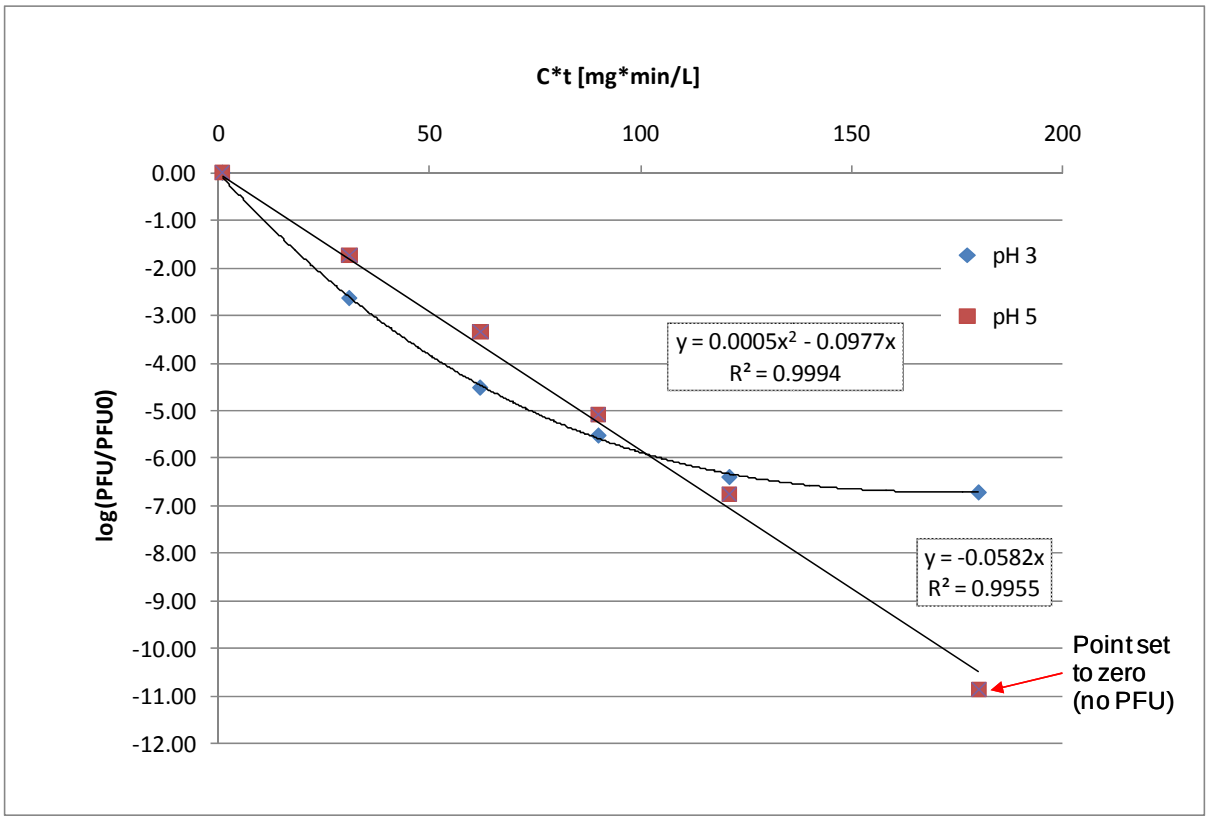


Figure 23: Comparison of disinfection kinetics in aggregated and mono-dispersed state in PBS (initial MS2 concentration 10^{11}PFU/ml)

As seen in the aggregation studies with the DLS (4.1.3), MS2 was expected to form larger aggregates at low pH in sodium chloride than in phosphate buffer. Thus, changes in disinfection kinetics were expected. Figure 24 confirms the hypothesis. Disinfection at pH 5 in NaCl (20 mM) has a comparable disinfection rate (k= 0.075) as in PBS at pH 5 (k= 0.058), while at pH 3 the disinfection rate is extremely slow (k= 0.0224). In this case, no tailing was observed, likely because virus concentrations were not low enough.

To estimate the state of aggregation, the b value in Grant's model (equation 11) was calculated by dividing the reaction constants and solving the Riemann zeta function. The resulting b value of 1.413 indicates a highly aggregated system. The observed aggregation

slopes are resumed in Grant (1995) and are localized between 1.3 for very aggregated and 4 for less aggregated systems. This is a conservative estimation, because in Grants model, the virus survival is measured with aggregated virus, while here we are working with dispersed virus in plate counting. Plated aggregated virus would make the line at pH 3 of Figure 24 less steep (less PFU with aggregates), and thus decrease the disinfection rate and increase the aggregation factor b in equation 11.

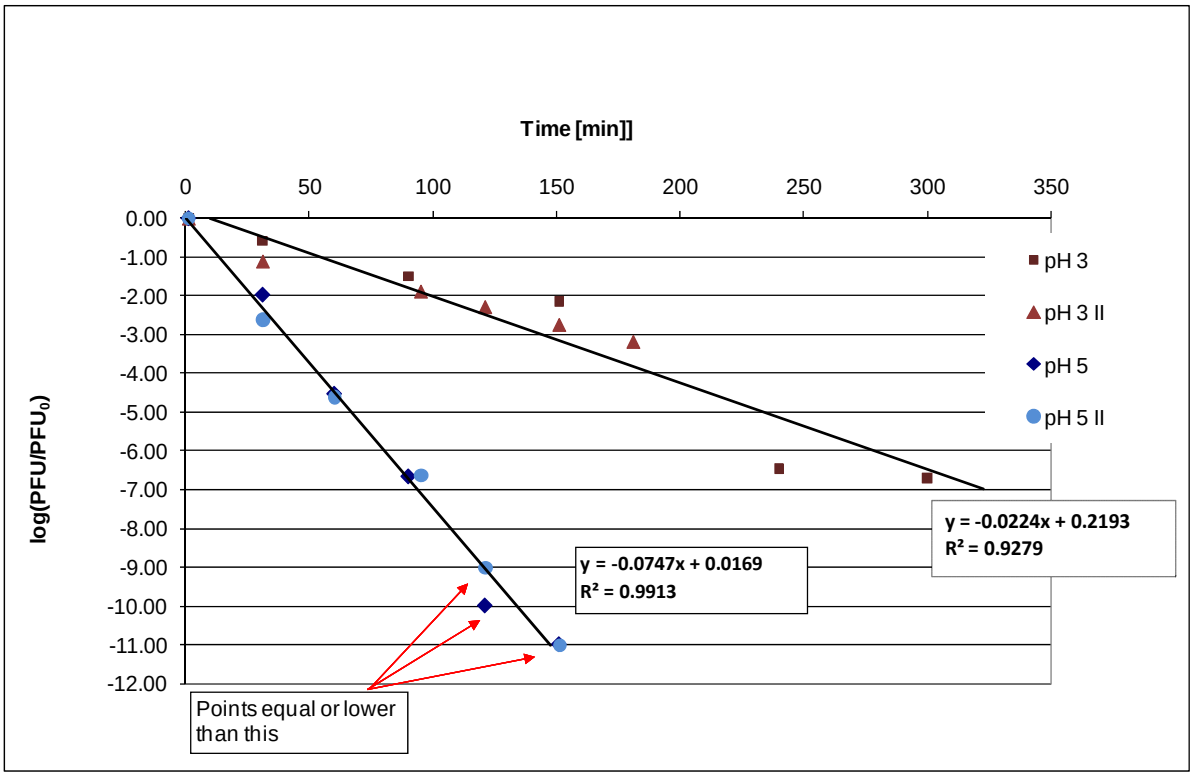


Figure 24: Comparison of disinfection kinetics in aggregated and mono-dispersed state in 20 mM NaCl (Initial concentration MS2 10^{11}PFU/ml)

5 Conclusion

This work demonstrates the importance of solution chemistry like ionic strength, pH and salt composition on the aggregation of viruses. MS2 aggregated when solution pH was less than 4 and ionic strength was less than 100 mM. Inactivation rates in PBS at pH 3 with UV disinfection were two times higher than in PBS at pH 7. Inactivation rates in 20 mM NaCl at pH 5 (dispersed) and pH 3 (aggregated) in PAA showed that aggregated viruses can be more resistant to chemical disinfection than dispersed viruses.

For the development of drinking water standards, aggregation has to be taken into account. Viruses which are known to aggregate under environmental conditions have to be tested in aggregated and dispersed state in order to account for effects of aggregation and adjust the disinfection dose.

Furthermore these results are important for the design of filters because efficiency could be overestimated for aggregated viruses.

6 Future work

Here we have clearly shown that MS2 is not easy to aggregate. In future studies, I recommend repeating this work with viruses that aggregate under existing environmental conditions and are easy to disperse (for example Reovirus or Poliovirus). Prior to choice of virus to be used in future studies the zeta potential should be determined in order to determine if its isoelectric point is in a reasonable pH range. Measurements with the DLS are faster than plating, so a screening for aggregates with DLS should be done before plating experiments are started.

It is not clear yet why the addition of $CaCl_2$ resulted in the loss of PFU. DLS results showed that the loss of PFU was not due to the effect of aggregation. It must therefore be due to inactivation. $CaCl_2$ addition should also be studied with varied pH, because near the pI it could aid in virus aggregation by compressing the double layer,

Stocks stored in NaCl and carbonate buffer should be tested by using the required buffer in the growth protocol of MS2 (3.1.2). The zeta potential (or electrophoretic mobility) should be measured with different ionic strengths to see if it has a major influence. Experiments of disinfection should be performed at varying pH, ionic strength and ionic composition to determinate the conditions for aggregation. All result should be classified in frequency of group sizes.

Size distribution of aggregates at low pH should be defined better than in this work. Even if differences in aggregation could be seen with DLS (i.e. phosphate buffer and acetic acid at pH 3), no detailed information of the aggregate sizes is available. It would be interesting to know if there are only few large particles or all the range of particles to explain effects like tailing as seen at low pH with phosphate buffer. More specified techniques should be applied, that can identify group sizes. Field- Flow Fraction can be used to separate particles prior DLS analysis and thus classify group sizes (Chuan 2008). Also techniques like Cryo-TEM can help in understanding the nature of these aggregates. It is more appropriated for aggregate analysis than TEM because there is no creation of artifacts as possible in negative staining. Analysis with Cryo could be difficult so if only few large particles are present.

Experiments of aggregation should be repeated with varying concentrations of virus, because aggregation can depend on it (Floyd and Sharp 1977).

The quantity of adsorption near the pI has to be determinate. A possibility is removing virus from the wall with beef extract.

The differences between disinfection at pH 3 and pH 7 with UV should be explained further, even if it seems like they are not related directly to aggregation. The mechanisms of UV-disinfection have to be investigated further and studies on ROS generation and quenching as well as on direct oxidative damage have to be performed.

Disinfection kinetics with peracetic acid showed a clear dependency on aggregation. These results have to be confirmed by comparing with other organism at the same pH, in order to confirm that there is no change in disinfection potential of peracetic acid between pH 3 and 5.

References

Abad, F. X.; R. M. Pinto; J. M. Diez and A. Bosch (1994), Disinfection of Human Enteric Viruses in Water by Copper and Silver in Combination with Low Levels of Chlorine, Applied and Environmental Microbiology 60, No 7, 2377-2383

Bisby, R.H.; Morgan C.G.; Hamblett I.; Gorman A.A. (1999), Quenching of singlet oxygen by Trolox C, ascorbate, and amino acids: Effects of pH and temperature, Journal of Physical Chemistry A 103, No 37, 7454-7459

Boedtker H., Gesteland R. (1975), Physical properties of RNA bacteriophages. In: Zinder ND, editor. RNA phages. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press; 1–28.

Butot, S.; T. Putallaz; C. Croquet; G. Lamothe; R. Meyer; H. Joosten; and G. Sánchez (2007), Attachment of Enteric Viruses to Bottles, Applied and Environmental Microbiology 73, No. 16, 5104–5110

Cherney, Daniel P.; Stephen E. Duirk; James C. Tarr and Timothy W. Collette (2006), Monitoring the Speciation of Aqueous Free Chlorine from pH 1 to 12 with Raman Spectroscopy to Determine the Identity of the Potent Low-pH Oxidant, Applied Spectroscopy 60, Number 7, 764-772

Chick H. (1908), An Investigation of the Laws of Disinfection, The Journal of Hygiene 8, No. 1, 92-158

Chuan, Yap P.; Yuan Y. Fan; Linda Lua; Anton P.J. Middelberg (2008), Quantitative Analysis of Virus-Like Particle Size and Distribution by Field-Flow Fractionation, Biotechnology and Bioengineering 99, No. 6, 1425-1433

Clevenger, T.; Wu, Y.; DeGruson, E.; Brazos, B.; Banerji, S. (2007), Comparison of the inactivation of Bacillus subtilis spores and MS2 bacteriophage by MIOX, ClorTec and hypochlorite, Journal of Applied Microbiology 103, No. 6, 2285-2290

Costello, M. Joseph (2006), Cryo-Electron Microscopy of Biological Samples Ultrastructural Pathology 30, 361–371

Crittenden, John C.; R. Rhodes Trussell; David W. Hand (2005), Water Treatment: Principles And Design, John Wiley & Sohn, 643-773

Date, Takayasu (1979), Kinetic Studies of the Interaction between MS2 Phage and F Pilus of Escherichia coli, Eur. J. Biochem. 96, 167-175

Davies, Michael J. (2003), Singlet oxygen-mediated damage to proteins and its consequences, Biochemical and Biophysical Research Communications 305, 761–770

Davies, Michael J.; Roger J.W. Truscott (2001), Photo-oxidation of proteins and its role in cataractogenesis, J. of Photochemistry and Photobiology B: Biology 63, 114–125

Davies-Colley, r. J.; A. M. Donnison; D. J. Speed; C. M. Ross and J. W. Nagels (1999), Inactivation of faecal indicator micro-organisms in waste stabilisation ponds: Interactions of environmental factors with sunlight, Wat. Res. 33, No. 5, 1220-1230

Delgado, A. V.; F. Gonzalez-Caballero, R. J. Hunter, L. K. Koopal, and J. Lyklema (2005), Measurement and interpretation of electrokinetic phenomena, IUPAC Technical Report, Pure Appl. Chem. 77, No. 10, 1753–1805

Eischeid, Anne C.; Joel N. Meyer and Karl G. Linden (2008), UV disinfection of adenoviruses: Molecular indications of DNA damage efficiency, published online ahead of print on 31 October 2008, Appl. Environ. Microbiol., doi:10.1128/AEM.02199-08

Ershov, B. G. and P. A. Morozov (2008), Decomposition of Ozone in Water at pH 4–8, Russian Journal of Applied Chemistry, 2008, Vol. 81, No. 11, pp. 1895–1898

Floyd, Roger; D. G. Sharp (1977), Aggregation of Poliovirus and Reovirus by Dilution in Water, Applied and environmental Microbiology 33, 159-167

Floyd, Roger; D. G. Sharp (1978a), Viral Aggregation: Effects of Salts on the Aggregation of Poliovirus and Reovirus at Low pH, Applied and environmental Microbiology 35, 1084-1094

Floyd, Roger; D. G. Sharp (1978b), Viral Aggregation: Quantitation and kinetics of the aggregation of Poliovirus and reovirus, Applied and environmental Microbiology 35, 1079-1083

Floyd, Roger; D. G. Sharp (1979), Viral Aggregation: Buffer Effects in the Aggregation of Poliovirus and Reovirus at Low and High pH, Applied and environmental Microbiology 38, 395-401

Galasso, G. J. and D. G. Sharp (1962), Virus particle aggregation and the plaque-forming unit, J. Immunology 88, 229-247

Galasso G. J. and D. G. Sharp (1965), Effect of Particle Aggregation on the Survival of Irradiated Vaccinia Virus, J. of Bacteriology 90, No. 4, 1138-1142

Galdiero, F. (1979), Adenovirus Aggregation and Preservation in Extracellular Environment, Archives of Virology 59, 99—105

Grabow, W. O. K, P. Couborough, C. Hilner, and B. W. Bateman (1984), Inactivation of hepatitis A virus, other enteric viruses and indicator organisms in water by chlorination, Water Sci. Technol. 17, 657-664.

Gordon R. Finch and Neil Fairbairn (1991), Comparative Inactivation of Poliovirus Type 3 and MS2 Coliphage in Demand-Free Phosphate Buffer by Using Ozone, Applied and environmental Microbiology 57, No. 11, 3121-3126

Grant, Stanley B. (1995), Inactivation kinetics of viral aggregates, J. of environmental engineering 121, 311-319

Hijnen, W.A.M.; E.F. Beerendonk; G.J. Medema (2006), Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review, Water Research 40, 3–22

Hejkal, Thomas W.; Flora Mae Wellings; Arthur L. Lewi and Paul A. Larock (1981), Distribution of Viruses Associated with Particles in Wastewater, Applied and environmental Microbiology 41, 628-634

Jensen, H.; Kent Thomas and D. G. Sharp (1980), Inactivation of Coxsackieviruses B3 and B5 in Water by Chlorine Applied and Environmental Microbiology 40, No. 3, 633-640

Kim, K. S. and D. G. Sharp (1966) Electron microscopic observations on the nature of vaccinia virus particle aggregation, The Journal of Immunology 97, No. 2, 197-202

Kitis, Mehmet (2004), Disinfection of wastewater with peracetic acid: a review, Environment International 30, 47– 55

Kohn, Tamar and Kara L. Nelson (2007), Sunlight-Mediated Inactivation ofMS2 Coliphage via Exogenous Singlet Oxygen Produced by Sensitizers in Natural Waters, Environ. Sci. Technol. 41, 192-197

Koivunen, J.; H. Heinonen-Tanski (2005), Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments, Water Research 39, 1519–1526

Kutter E., Sulakvelidze A. (2004), Bacteriophages Biology and Applications, CRC Press, Introduction p 1

Kuzmanovic, Deborah A.; Ilya Elashvili; Charles Wick; Catherine O'Connell and Susan Krueger (2006), The MS2 Coat Protein Shell is Likely Assembled Under Tension: A Novel Role for the MS2 Bacteriophage A Protein as Revealed by Small-angle Neutron Scattering; J. Mol. Biol. 355, 1095–1111

Langlet, J.; F. Gaboriaud, C. Gantzer (2007) Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage, J. of Applied Microbiology 103, 1632-1638

Langlet, Jérémie; Fabien Gaboriaud; Christophe Gantzer and Jérôme F. L. Duval (2008a), Impact of Chemical and Structural Anisotropy on the Electrophoretic Mobility of Spherical Soft Multilayer Particles: The Case of Bacteriophage MS2, Biophysical Journal Volume 94, 3293–3312

Langlet, Jérémie; Fabien Gaboriaud; Jérôme F.L. Duval and Christophe Gantzer (2008b), Aggregation and surface properties of F-specific RNA phages: Implication for membrane filtration processes, Water Research 42, Issues 10-11, 2769-2777

Maillard, J.-Y.; Beggs, T.S.; Day, M.J.; Hudson, R.A. and Russell, A.D. (1994), Effect of biocides on MS2 and K Coliphages, Applied and Environmental Microbiology 60, No 6, 2205-2206

Mayo, M. A. and I. M. Roberts (1979), Some Effects of Buffers on the Infectivity and Appearance o f Virus Inocula used for Tobacco Protoplasts, J. gen. Virol. 44, 691-698

Narang, H. K. and A. A. Codd (1981), Frequency of Preclumped Virus in Routine Fecal Specimens from Patients with Acute Nonbacterial Gastroenteritis, Journal Of Clinical Microbiology 13, No 5, 982-988

Ohshima, Hiroyuki (1994), A simple expression for Henry's Function for the retardation Effect in Electrophoresis of spherical, colloidal particles, J. of Colloid and Interface Sci. 168, 269-271

Ohshima, Hiroyuki (2007), Electrokinetics of soft particles (review), Colloid Polym. Sci. 285, 1411-1421

Penrod, Samuel L.; Terese M. Olson, and Stanley B. Grant (1996), Deposition Kinetics of Two Viruses in Packed Beds of Quartz Granular Media, Langmuir 12, 5576-5587

Rahn, Ronald 0. (1997), Potassium Iodide as a Chemical Actinometer for 254 nm Radiation: Use of Iodate as an Electron Scavenger Photochemistry and Photobiology 66(4): 450-455

Redman, J. A.; S. B. Grant, T. M. Olson, M. E. Hardy, and M. K. Estes (1997), Filtration of Recombinant Norwalk Virus Particles and BacteriophageMS2 in Quartz Sand: Importance of Electrostatic Interactions, Environ. Sci. Technol. 31 (12), 3378-3383

Schaldach, C.M.; Bourcier, William L.; Shaw, Henry F.; Viani; Brian E.; Wilson; W.D. (2006), The influence of ionic strength on the interaction of viruses with charged surfaces under environmental conditions, Journal of Colloid and Interface Science 294, 1–10

Sharp, D. G.; R. Floyd and J. D. Johnson (1975), Nature of the Surviving Plaque-Forming Unit of Reovirus in Water Containing Bromine, Applied Microbiology 29, 94-101

Simonet, Julien and Christophe Gantzer (2006), Inactivation of Poliovirus 1 and F-Specific RNA Phages and Degradation of Their Genomes by UV Irradiation at 254 Nanometers, Applied and environmental Microbiology 72, No. 12, 7671–7677

Teunis, Peter F.M; Christine L. Moe; Pengbo Liu; Sara E. Miller; Lisa Lindesmith; Ralph S. Baric; Jacques Le Pendu, and Rebecca L. Calderon6 (2008), Norwalk Virus: How Infectious is It?, Journal of Medical Virology 80, 1468–1476

Thurman, R.N.; C.P. Gerba (1988), Molecular mechanism of viral inactivation by water disinfectants, Advances in Applied Microbiology 33, 75-105

Thurston-Enriquez, Jeanette A.; Charles N. Haas; Joseph Jacangelo and Charles P. Gerba (2003) Chlorine Inactivation of Adenovirus Type 40 and Feline Calicivirus, Applied and environmental Microbiology 69, No. 7, 3979–3985

Thurston-Enriquez, Jeanette A.; Charles N. Haas; Joseph Jacangelo and Charles P. Gerba (2005), Inactivation of Enteric Adenovirus and Feline Calicivirus by Chlorine Dioxide, Applied and environmental Microbiology 71, No 6, 3100–3105

Wallis, Craig and Joseph L. Melnick (1967), Virus Aggregation as the Cause of the Non-neutralizable Persistent Fraction, Journal of Virology 1, 478-488

Yuan B., Mai Pham, and Thanh H. Nguyen (2008), Deposition Kinetics of Bacteriophage MS2 on a Silica Surface Coated with Natural Organic Matter in a Radial Stagnation Point Flow Cell, Environ. Sci. Technol., 42 (20), 7628-7633

Young, D. C. and D. G. Sharp (1977), Poliovirus Aggregates and Their Survival in Water, Applied and environmental Microbiology 33, No. 1, 168-177

Watson, H.E. (1908), A note on the variation of the rate of disinfection with change in the concentration of the disinfectant. Journal of Hygiene 8, 536 542

Zerda, K.S. and Gerba, C.P. (1984), Agarose isoelectric focusing of intact virions. J Virol Methods 9, 1–6

APPENDIX

List of materials

Name	Specification	Producer
50 ml tubes	50 ml, 114x28mm	Sarstedt
Acetic Acid (glacial)		Merck
Acetic Acid Buffer	20mM aceticacid in nanopore water; pH adjusted with HCL and NaOH	
Agar Agar	granular	Merck kGaA
BactoTM Tryptone	Pancreatic Digest of Casein	BD
Bleach	Comercial Bleach	
Calcium Chloride	CaCl2 dihydrate for Analysis	Acros organics
Carbonate Buffer	1mM NaHCO3; 10mM NaCl	
Centrifuge	5417R	Eppendorf
Cuvette for DLS	120 ul	
D(+) Glycose	anhydrous, ACS reagent	Acros organics
Dynamic Light Scattering	Zetasizer Nano ZS	Malvern Instruments Ltd.
Eppendorf	1.5 ml	Nerbe Plus
Filter for size exclusion (0.45, 0.22 and 0.	Durapore®Membrane Filters	Millipore
Filter 0.1um for sterilization	Millex-VV Syringe driven filter unit	Millipore
Filter 50nm	IsoporeTM Membrane Filters	Millipore
Filter cartridge for 50nm filters	Swinnex Non-sterile	Millipore
Filter centrifuge	Microcon Ultracel YM 100	Amicon bioseparations
Glucose	Polyethylene Glycol 6000	Fluka
Grid for TEM	Cupper	Agar company
Hydrochloric Acid	Standard Solution	Acros organics
Nanopore	Milli Q Water system	Milli Q
Parafilm	Parafilm "M" Laboratory film	Pechiney
Peracetic Acid Test	Colorometric with Test Strips 5-50mg/L	Merckoquant
pH Meter	780 pH Meter	Ohm Metrohm
pH paper	Universalindikatorpapier	Michael-Nagel (MN)
Photometer	UV-2550	Shimadzu
Pipette		Rainin; Thermo scientific
Sodium Bicarbonate		Acros organics
Sodium chloride		Acros organics
Sodium Hydroxide		Acros organics
Sodium Phosphate	Monobasic	Acros organics
TEM	CM 100	Philips
Virus Dilution Buffer	5mM PO_4; 10mM NaCL	
Vortexer	Vortex- Genie 2	Scientific Industries

Theory of DLS

The zetasizer is equipped with a laser attenuator and an avalanche photodiode detector. It measures the changes in intensity of scattered light at an angle of 1730. The intensity change is shown on a correlation curves and related to the diffusion coefficient. The Stokes- Einstein converts diffusion to the size of particles. The resulting size distributions are shown in intensity, volume or number distributions. In the intensity distributions, larger particles are more present because they scatter much more light than small particles (proportional to the sixth power of its diameter, Rayleigh's approximation). The resulting volume distribution thus emphasize smaller particle. Number distribution emphasize even more than the volume distribution ($\frac{4}{3}\pi r^3$, Volume of a sphere). Electrophoretic mobility was measured by laser Doppler electrophoresis. The rate of change of the phase shift between the scattered light and a reference beam is correlated to the particle velocity.

Dilution series and plating

For virus counts, the virus-containing sample was diluted several times in order to plate a countable number of plaque forming units (PFU). Normally 1:100 and 1:10 dilutions were used in a series. For 1:10 dilutions, 900 µl Buffer was filled in an eppendorf container and 100 µl of the sample was added. For 1:100 dilutions, 990 µl buffer was spiked with 10 µl sample. For plating, the soft agar tubes were melted in a water bath at 95 ˚C and then transferred to a water bath at 56 ˚C to cool down.

Near a Bunsen burner flame, into every soft agar tube, 100 µl of *E.Coli* (prepared the night before) and 100 µl of the sample were added. Then the soft agar was vortexed and dispersed carefully on the hard agar plate. The pipette tip of the sample was changed for every dilution or sample. The plates were incubated over night at 37 ˚C and the plaques on the *E.Coli* lawn were then counted.

Contamination

Contamination was a major problem during the study. Plating was difficult, because practically all plates had a big amount of PFU.

In order to avoid contamination, following precautions were adopted

- Cleaning of the table with Ethanol (70%) and Bleach (10%) before and after every working step
- Autoclaving of all material used, especially pipette tips, buffer solutions and Eppendorf tip containers
- Cleaning of pipettes with bleach before using
- Working near the flame at every crucial step, especially for the preparation of buffer solutions, dilution series and plating
- Wearing of gloves and coat
- Change of gloves before every crucial step, especially preparation of buffer solutions, dilution series and plating
- After contamination happened, all solutions were again autoclaved or if autoclaving was not possible, thrown out and replaced