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Master thesis

Disinfection of *Enterobacteria phage MS2* by Chlorine dioxide as related to the Tailing phenomenon

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Contents

Abstract

Inactivation of microorganisms is one of the major issues that guarantees global health. In particular, the disinfection of water is of crucial importance for a secured drinking water supply. This thesis investigates a particular deviation of disinfection kinetics, known as Tailing phenomenon, that appears in case of the disinfection of *Enterobacteria phage MS2* by Chlorine dioxide (ClO_2). Although Tailing has been observed in several studies for different organisms it is still unclear why the process occurs. Three hypotheses were tested to disclose this Tailing phenomenon: (1) Loss of biocidal properties of the experimental solution, (2) Aggregation of virus particles and (3) Existence of a resistant virus subpopulation. The disinfectant ClO_2, the indicator virus *Enterobacteria phage MS2* and the belonging host *Escherichia coli* were used in all disinfection experiments performed. To verify Hypothesis 1 two differnet experiments were perfomed. First, the same amount of virus was added to the experimental solution in the time range when Tailing started. Second, the Disinfection by-product (DBP) Chlorite (ClO_2^-) was added before starting. One run was conducted with a full dose and a second run with a half dose of the produced ClO_2^- in the course of a disinfection experiment. To verify Hypothesis 2 beside disinfection experiments also Dynamic Light Scattering (DLS) measurements have been performed. The results of the DLS measurements showed no aggregate formation in the case of a standard disinfection experiment. Besides the physical – filtration, sonication before starting and in the time range of Tailing – and chemical treatment of the experimental solution – Chloroform ($CHCl_3$) extraction, buffer with a fifty times higher Sodium chloride (NaCl) concentration – a lower virus concentration has also been used to influence the course of disinfection. To verfiy Hypothesis 3, two different experiments were performed. In both cases, a standard disinfection experiment was performed until the time range when Tailing started. In one case, the experimental solution was washed for five times and then the experiment was restarted again. In the other case the concentration of the added ClO_2 solution was increased approximately fivefold. For all performed experiments the received disinfection curves possessed Tailing behaviour. Consequently all three proposed hypotheses had to be rejected.
However, the results of Hypothesis 3 – especially for the experiment with a washing pause – constitute the starting point for further investigations that finally disclosed the mystery Tailing phenomenon.

Zusammenfassung

Die Inaktivierung von Mikroorganismen wird als zentraler Punkt zur Gewährleistung von globaler Gesundheit angesehen. Besonders die Desinfektion von Wasser hat eine entscheidende Bedeutung um eine sichere Trinkwasserversorgung zu ermöglichen. Diese Arbeit untersucht eine spezielle Abweichung der Inaktivierungskinetik, die bei der Desinfektion von *Enterobakteriophage MS2* mit Chlordioxid (ClO_2) auftritt und als Tailing Phänomen bezeichnet wird. Drei Hypothesen wurden überprüft um dieses Tailing Phänomen zu untersuchen: (1) Verlust der bioziden Wirkung der Versuchlösung, (2) Aggregation von Viruspartikeln und (3) Existenz einer resistenten Virussubpopulation. Das Desinfektionsmittel ClO_2, der Indikatorvirus *Enterobakteriophage MS2* und der entsprechende Wirt, *Escherichia coli*, wurden in allen durchgeführten Desinfektionsexperimenten verwendet. Um Hypothese 1 zu überprüfen wurden zwei verschiedene Experimente durchgeführt. Einerseits wurde im Startbereich von Tailing erneut die gleichen Menge an Virus zur Versuchslösung zugegeben, andererseits wurde vor dem Beginn des Experiments, das während eines Laufes entstehende Desinfektionsnebenprodukt Chlorit (ClO_2^-) zugegeben. Ein Lauf wurde mit der vollen Dosis und ein zweiter Lauf mit der halben Dosis ClO_2^- durchgeführt. Um Hypothese 2 zu überprüfen wurden neben Desinfektionsexperimenten auch Messungen der dynamischen Lichtstreuung (DLS) durchgeführt. Bei den DLS Messungen im Zuge eines Standarddesinfektionsexperiments konnte keine Aggregatbildung nachgewiesen werden. Neben physikalischer – Filtration, Ultraschallbehandlung vor Beginn und im Startbereich von Tailing – und chemischer Behandlung der Versuchlösung – Chloroform($CHCl_3$)-Extraktion, Puffer mit fünzigfach höherer Natriumchlorid(NaCl)-Konzentration – wurde auch eine geringer konzentrierte Viruslösung eingesetzt, um den Verlauf der Desinfektion zu beeinflussen. Um Hypothese 3 zu überprüfen, wurden zwei verschiedene Experimente durchgeführt. In beiden Fällen wurde ein Standarddesinfektionsexperiment durchgeführt und jeweils im Startbereich von Tailing unterbrochen. Einmal wurde die Versuchslösung fünfmal gewaschen und das Experiment wieder gestartet. Im anderen Fall wurde die Konzentration der zugegebenen ClO_2 Lösung in etwa um das Fünffache erhöht. Schlussendlich mussten alle drei aufgestellten Hypothesen verworfen werden, da alle ermittelten Desinfektionskurven Tailing-Verhalten anzeigten.
Jedoch wurden die erhaltenen Ergebnisse der Überprüfung von Hypothese 3 zum Ausgangspunkt für weitere Untersuchungen, die schließlich zur Aufklärung des Tailing Phänomens führten.

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1 Introduction

1.1 Aim of the study

The overall aim of this study is to gain broader knowledge of the phenomenon that is known in the academic community as Tailing. This phenomenon describes the occurance of incomplete inactivation that has been observed in the course of several disinfection processes. As already mentioned, one fact of Tailing involves the incomplete inactivation of possible pathogens, but another consequence is that the established disinfection models – which are used to determine the necessary treatment properties – no longer fit.
Due to the globally important issue of safe drinking water supply which is also influenced by Tailing, this thesis studies the consequences in the case of drinking water treatment.
As viruses are a major health issue in drinking water supply, the investigations in this study focus on them. ClO_2 was chosen as the disinfectant, because Tailing has been observed when it interacts with viruses. Therefore, indicator organism *Enterobacteria phage MS2* (*MS2*) and its well-known, belonging host *Escherichia coli* (*E. coli*) have been selected. Three different proposed hypotheses were tested by performing disinfection experiments, and Double-layer plaque assay and Dynamic DLS were used to determine the results. For a detailed time line see fig. 1.

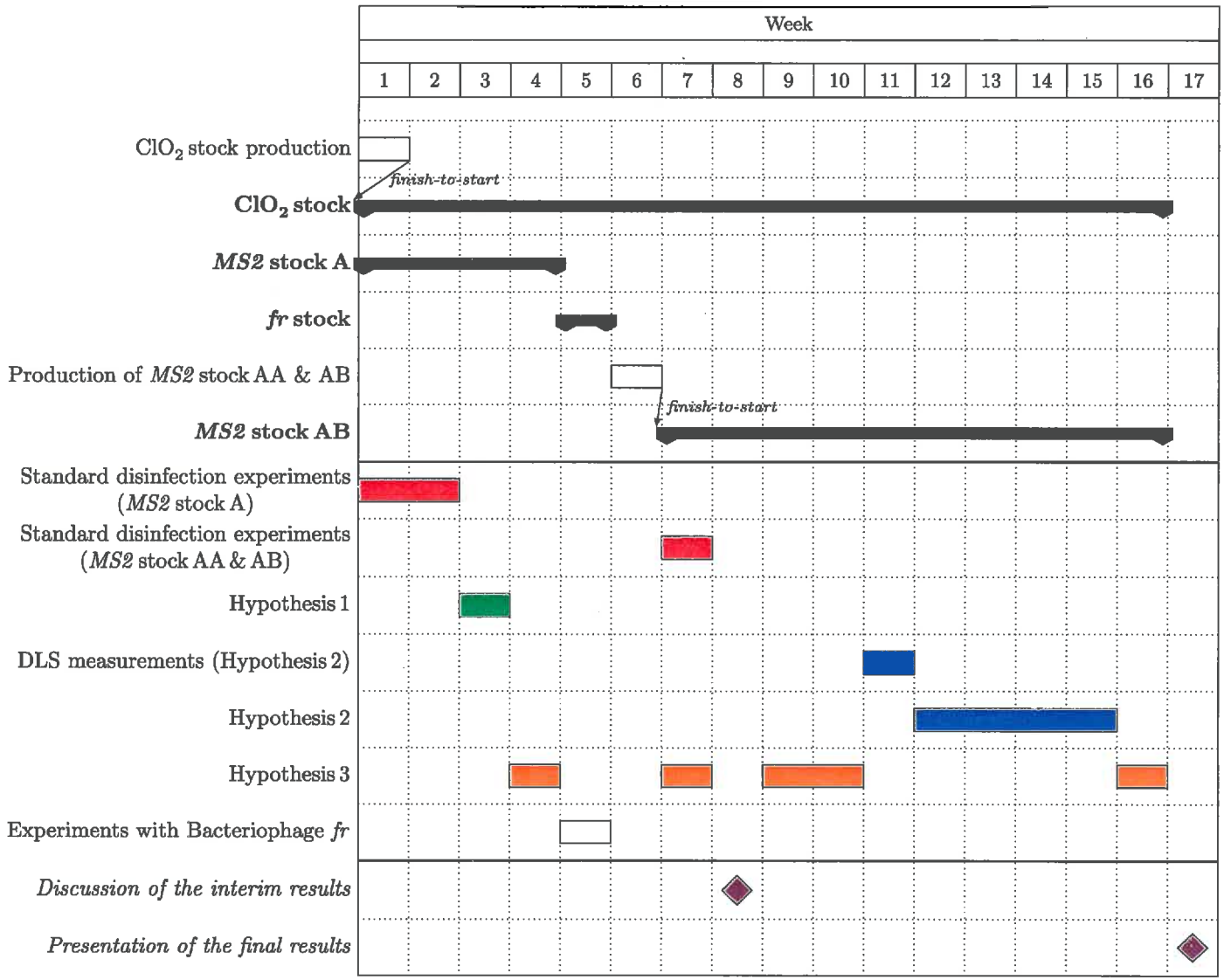


Figure 1: Time line of the study

1.2 State of the art

1.2.1 Disinfection

The World Health Organisation (WHO) lists disinfection along with filtration, coagulation, flocculation, sedimentation, and protection of source water and the distribution system as a basic part of the concept of multiple barriers. This is because that concept is considered as the cornerstone of safe drinking water production([27]). The term "disinfection" is defined as following:

„Maßnahme, die durch Abtöten, Inaktivieren bzw. Entfernen von Mikroorganismen (Bakterien, Viren, Pilze, Protozoen) eine Reduzierung der Keimzahl erreicht, so dass von dem desinfizierten Material keine Inf.[ektion] mehr ausgehen kann;" (Bach, 2007, 415)

"An action which reduces germs by mortification, inactivation or removal of microorganisms (bacteria, viruses, fungi, protozoa) so that no infection of the disinfected material can occur."

To achieve inactivation, a functioning system of principal factors have to be established. In addition to a disinfectant and the concentration of the disinfectant, the contact time, temperature and pH are also such factors. In order to develop a disinfection process that inactivates as many microorgansims as possible, the composition of the individual factors have to be explored. Ideally, the disinfectant that is used in the final application should be tested under the same environmental conditions that are present in the final application. However, in many cases this can cause serious risk for the health of the investigatory staff, and the effort to investigate all occuring species can easily extends far beyond the possibilities. Therefore, so-called indicator species are used for experiments. These species represent a broad range of different organisms with similar properties like structure, morphology, size, as well as similar behaviour in their typical habitat. Most importantly, the big advantage of these indicator organisms is that they are not harmful to humans, can easily be cultivated and propagated, and also are very well investigated.
Famous examples for indicator organism are *Mus musculus* for mammals, *Arabidopsis thaliana* for plants, *Saccharomyces cerevisiae* for yeasts, *E. coli* for bacteria and *MS2* for bacteriophages. The latter two have been used for several experiments in this thesis.

Frequently used disinfectants are halogens like Chlorine (Cl_2) and ClO_2, Ozone (O_3), Ultraviolet (UV) radiation and heat. In this thesis the effect of Chlorine Dioxide has been investigated.

Considering the danger of pathogens, it is of vital importance to be able to predict the outcome of a disinfection process. Or in other words, how many potential pathogens can be killed in a distinct time period.
Therefore different models and theories have been postulated. The principal model used for linear disinfection curves is the CHICK-WATSON model ([30]). It expresses the rate of inactivation of a pathogen by a first-order chemical reaction and is shown in eq. 1 and eq. 2.

$$\frac{N_t}{N_0} = e^{-kt} \tag{1}$$

or in antilogarithmic form:

$$\ln \frac{N_t}{N_0} = -kt \tag{2}$$

For a description of N_t, N_0, k and t see "List of Symbols" in the Appendices.

Plotting the logarithm of the survival rate N_t/N_0 on the y-axis against the time duration of the disinfection procedure results in a linear line (see fig. 2), if the disinfection follows an ideal behaviour. Another possibility is to plot the y-axis in logarithmic display.

It is in the nature of things that the way humans describe the world never fits the whole issue. So is it that several different deviations of a first-order kinetic have been observed. These deviations include Shoulder Curves and Tailing Curves – also referred as Tail, Tailing off or Biphasic Curves – and usually they can not be described by the CHICK-WATSON model in a proper way. That is why CERF proposed a two-fraction model that could be used to describe Biphasic Curves (an example is shown in fig. 8).

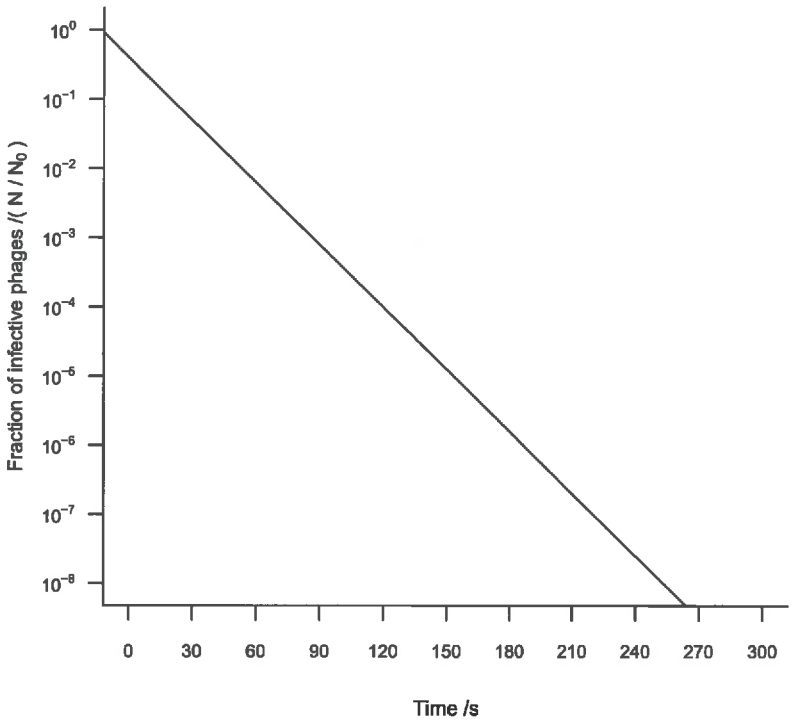


Figure 2: Resulting curve of a disinfection that follows ideal behaviour

1.2.2 Tailing phenomenon

These Tailing Curves have been observed by several authors and for different organisms like bacteria ([23]), fungi spores ([16]), viruses ([10]) and bacteriophages ([22]), but no consensus about the origin have been reached until today. In a review about Tailing, the author declared the phenomenon in the following way:

"People who have observed tails or who have considered the question, either accept tails as facts or reject them as artefacts." (Cerf, 1977, 3)

CERF distinguishes the acceptor group further into followers of a mechanistic and a vitalistic concept.
The vitalistic theory explains Tailing as due to heterogeneous nature within the population. That means the population possesses different degrees of resistance against a disinfectant, and this difference is permanent.
In contrast, the mechanistic theory assumes that the population is of homogeneous nature and the process of destruction is a close analogy to a chemical reaction. Therefore only a distinct amount of molecules of the disinfectant and the disinfected substances are capable of participating in the disinfection reaction, due to their position.

Whatever the reason for Tailing is, its occurance could result in incomplete inactivation and trigger crucial health problems for humans.

1.3 Chlorine Dioxide

Chlorine Dioxide was first discovered in 1811 by DAVY who called it Euchlorine ([12]). It is one of the few compounds that exists almost entirely as monomeric free radicals and is soluble in Water (H_2O), as it forms a greenish-yellow solution ([2]). At Room temperature (RT) it exists as a greenish-yellow to orange gas and is strongly oxidizing ([13]). Furthermore the achieved ClO_2 concentrations in H_2O lie in a range between 0,1 mg/L to 0,5 mg/L. Due to such low concentrations no explosive hazard in a possible available gas phase can develop ([1]). Additional properties are given in the following list ([20], [31], [2]):

- IUPAC-Name: Chlorine Dioxide
- Formula: ClO_2
- CAS Number: **10049-04-4**
- IUPAC Standard InChIKey: OSVXSBDYLRYLIG-UHFFFAOYSA-N
- Molecular weight: 67.452
- Meltingpoint: $-59\,°C$
- Boilingpoint: $11\,°C$
- Density: $2.757\ g\,mL^{-1}$
- Molar (decadic) absorption coefficient (ε): $1250\,dm^3\,mol^{-1}\,cm^{-1}$
- Oxidation state: Chlorine (IV)
- Oxidation number: 4
- Additional names: Alcide, Anthium dioxcide, Chlorine(IV)oxide, Chlorine oxide, Chlorine peroxide, Chloroperoxyl, Chloryl radical

The chemical structure of ClO_2 is shown below:

$$\cdot O{=}Cl{=}O \cdot$$

For the production of ClO_2 for drinking water treatment Sodium chlorite ($NaClO_2$) is used (see eq. 3).

$$2\,NaClO_2 + Cl_2 \longrightarrow 2\,ClO_2 + NaCl \tag{3}$$

For the chemical name of the given chemical formulas ($NaClO_2$ Cl_2, ClO_2, NaCl) see "List of Abbreviations" in the Appendices.

During the production of ClO_2 a decomposition into two major DBPs ClO_2^- and Chlorate (ClO_3^-) ([2]) can occur. Per mg ClO_2 comsumed or applied the amount of produced ClO_2^- lies about 0.5–0.7 mg (50–70%) and for ClO_3^- at approximately 0.3 mg (30%) ([3]). The chemical equation of the decomposition is shown in eq. 4.

$$2\,ClO_2 + H_2O \longrightarrow ClO_2^- + ClO_3^- + 2\,H^+ \tag{4}$$

For the chemical name of the given chemical formulas (ClO_2 H_2O, ClO_2^-, ClO_3^-, H^+) see "List of Abbreviations" in the Appendices.

As a disinfectant, ClO_2 is considered a good alternative to Cl_2 due to the fact that, contrary to Cl_2, no organohalogen by-products like Trihalomethaness (THMs) are built. Another advantage compared to Cl_2 is that no reaction with Ammonia (NH_3) takes place and therefore no loss of the virucide properties can happen if NH_3 is present.
Disadvantages include the decomposition into ClO_2^- and ClO_3^- as well as the formation of some organic DBPs like formaldehyde, acetaldehyde and acetone ([2]).
Additional favourable properties of ClO_2 are that it removes taste and odor compounds, that it is a good bactericide and viricide, that it is unaffected by pH variations in drinking water, and that it is equal or superior to Cl_2 on a mass-dose basis. ([1]).

In case of *MS2*, it has been shown ([39]) that ClO_2 just attacks the A protein and the capsid of *MS2* but causes no genome damage or inhibition of the replication function.

1.4 Bacteriophages

Bacteriophages are viruses that use bacteria as hosts. As viruses they propagate in a parasitic way. That means that the presence of an appropriate host is essential. Whereby it should be noted that the interaction between the virus and the host is very specific. Many times, just a single species is suitable as a host. The mentioned specific interactions can be very different but a few essential functions (Entrance; Transcription, Translation and Replication; Assembly of progeny virus particles; Egress) can be identified and seen as the basic skills each virus has to possess to be able to propagate. In order to achieve these essential functions, a virus must be able to perform the following steps:

1. Adsorption of the virion on the host
2. Penetration of the whole virion or at least of the nucleic acid (NA)
3. Replication and Transcription of the viral NA using the host metabolism and sometimes optional virus based proteins
4. Translation of capsid proteins
5. Self-assembly of the progeny virus particles
6. Egress of the new virus generation

1.4.1 *Enterobacteria phage MS2*

MS2, an indicator organism for enteric viruses, was first discovered by Alvin John CLARK in 1961 ([11]) and sequenced in 1976 ([15]), which represented the first genome ever sequenced. It is a member of the Levivirus genus and uses *E. coli* as a host. The entry occurs via F-pili or polar pili of the *E. coli*. The genome replication takes place in the Cytoplasm and is catalysed by a heterotetramer Replicase that is composed by three host proteins and one phage-encoded subunit. As *MS2* is a positive-sensed single-strand Ribonucleic Acid (ssRNA), the genome can serve as messenger Ribonucleic Acid (mRNA) for the direct translation of the viral proteins. To convert ssRNA into double-strand Ribonucleic Acid (dsRNA), the replicase forms the complementary negative-sensed ssRNA. The dsRNA is then used for the production of the genomic ssRNA of the next virus generation. The site of virion assembly takes also place in the Cytoplasm. The egress of the new built phages is obtained by bacteriolysis. One infection cycle of *MS2*, beginning with the entry and ending with lysis takes approximately 1 h ([32]). The whole genome encodes just four proteins:

- Maturation protein
- Coat protein
- Lysis protein[1]
- Replicase protein

The virion (see fig.3) is built up by 180 coat proteins, one maturation protein and one copy of genomic Ribonucleic Acid (RNA).

[1]Was one of the first examples of an overlapping gene

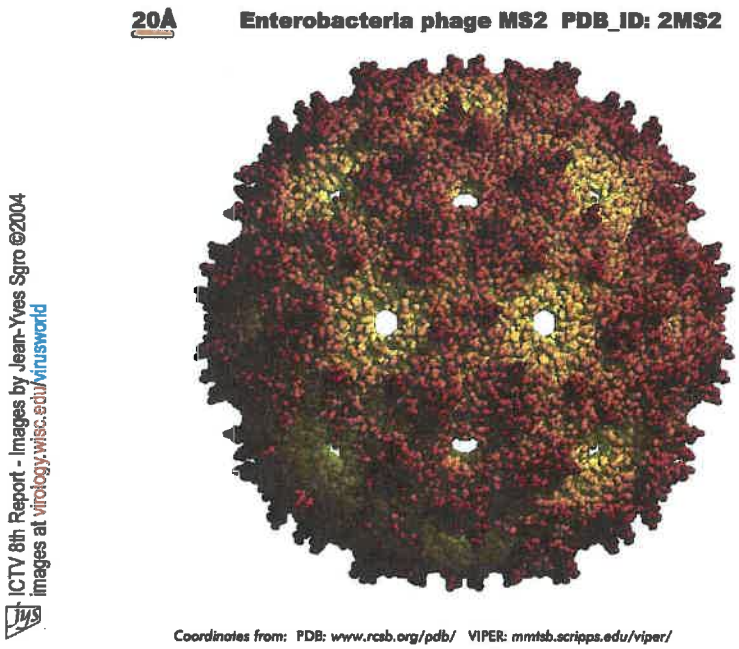


Figure 3: Virion structur of *Enterobacteria phage MS2*(Golmohammadi et al., 1993, ©)

For further information about *MS2*, see the folowing list ([32],[33]):

Classification:

- Class (regarding to Baltimore classification system): V
- Genus: Levivirus

Virion:

- Morphology: Icosahedral
- Envelope: No
- Diameter: 26 nm
- Molecular weight: 3.6×10^{-6}
- Isoelectric point (pI): 3.9

Genome:

- Nucleic acid: RNA
- Strandedness: Single-stranded
- Polarity: Positive-sense
- Configuration: Linear
- Size: 3569 nucleotides
- Guanin (G) + Cytosin (C) content: 50–52%
- Open reading frames: 4

Additional names: Bacteriophage *MS2*, *MS2* phage, *MS2*

1.4.2 Method to determine Bacteriophages

For the quantification of infective bacteriophages in (water) samples the so called "Double-layer plaque assay" [24] is used.
This method can be applied to F-specific RNA bacteriophages like *MS2* and can be used to prove the presence of such bacteriophages in water samples, which can help indicate contaminations with waste water that contains

human or animal faeces.

The decisive part of the method is the growing of the virus in the presence of the regarding host and a culture medium. In the case of *MS2* and *E. coli*, this results in a bacteria layer in the dish that is interrupted by clear spots, or so called plaques. Each plaque represents per definition a single infective virus particle – also called plaque forming unit (pfu) – that infected an *E. coli* cell and triggered its lysis. The descendent virus particles lyse further bacterial cells. Depending on the time duration, further descendents can continue with the process, leading to a clear spot in the bacteria layer.

1.5 *Escherichia coli*

It is the best known model organism in the world and was first described in 1885 by Theodor Escherich [14]. A few properties can be seen in the following list ([29]):

Classification (according to Bergey's Manual of Sytematic Bacteriology) :

- Domain: Bacteria
- Class: III. Gammaproteobacteria
- Family: I. Enterobacteriaceae

Morphology:

- Shape: Rod
- Size: $1 \times 2\,\mu$m
- Volume: $2\,\mu\text{m}^3$

Other properties:

- Metabolic physiology: Chemoorganotroph
- Respiration: Facultative aerobe
- Gram: Negative
- Catalase: Positive
- Oxidase: Negative

As the model organism of choice, the genome of several strains of *E. coli* have already been sequenced, such as strain K-12 ([6]) or strain O157:H7 ([35]). It is abundant in the human intestinal tract and probably plays an important role during the ingestion of food, because of its capability to produce vitamines – mainly Vitamin K. The wildtype strains are very frugal and can grow on a wide range of carbon and energy sources ([29]).

Most strains of *E. coli* are not pathogens, but some pathogenic strains have been discovered. They are classified due to the toxins they built like ETEC (Enterotoxigenic Escherichia coli), EHEC (Enterohaemorrhagic Escherichia coli) or STEC (Shiga Toxin-producing Escherichia coli). Some of them represent one of the biggest health problems in the Third World.

1.6 UV/Vis Spectroscopy

UV/Vis Spectroscopy is a very old and well-known instrumental method. The name arrives from the measured range of the electromagnetic spectrum. The UV stands for the ultraviolet range and Visible (Vis) for the visible range. For more details, see fig. 4.

The measuring principle is based on the interaction between electromagnetic radiation and matter – like atoms, molecules or ions – and can lead to different phenomena (e.g. absorbance, emission, reflexion). As absorbance is of particular importance for this thesis, it will be described in more detail. An absorption event occurs if an electron becomes transferred to a higher energy state. Therefore, a distinct amount of energy has to be available to be transferred from the irradation of electromagnetic radiation onto the sample substance, leading to an energy loss of the radiation. By comparing the incident and transmitted radiation, the transmittance (eq. 5) or absorbance (eq. 6) – two values that describes such absorption events – can be calculated. ([18])

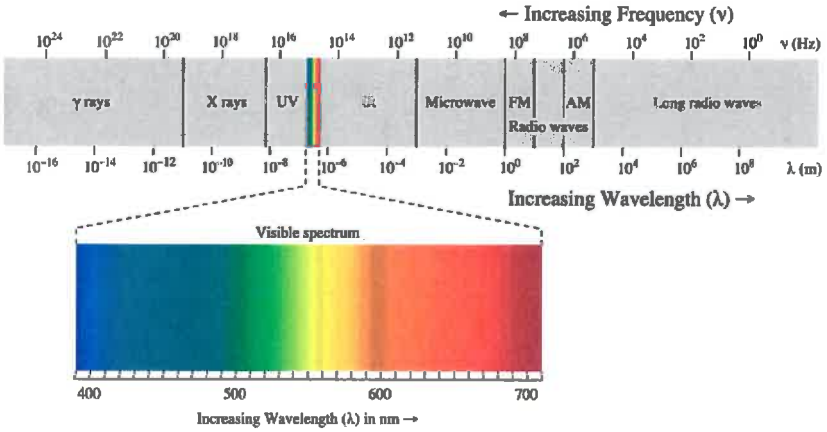


Figure 4: The electromagnetic spectrum (Zedh, 2007, © BY-SA 1.0)

$$T(l) = \frac{P_\lambda}{P_\lambda^0} \tag{5}$$

$$A(\lambda) = log_{10}\left(\frac{P_\lambda^0}{P_\lambda}\right) = -log_{10}\, T(\lambda) \tag{6}$$

For a description of $T(l)$, P_λ, P_λ^0 and A see "List of Symbols" in the Appendices.

For quantitative determinations UV/Vis Spectroscopy can be also used. In this case it is also called Photometry. The underlying basis are the observations that have been made by BOUGUER who noticed the relation between layer thickness and light transmittance ([7]), LAMBERT who found out that this is an exponential relation ([25]) and BEER who discovered the dependence of the absorbance on the concentration ([5]).
These observations are summarized in an equation that is called the BEER-LAMBERT-BOUGERT law which is often abbreviated as LAMBERT-BEER law ([8]). It is shown in eq. 7 and describes the relationship between the absorbance, layer thickness, concentration and molar (decadic) absorption coefficient – a substance dependent value. If the layer thickness and molar adsorption coefficient is known, the concentration of a sample can be calculated by measuring the absorbance.

$$A(\lambda) = \varepsilon(\lambda)\, c\, l \tag{7}$$

For a description of A, ε, λ, c and l see "List of Symbols" in the Appendices.

The basic parts for an UV/Vis Spectrometer are:

- Light source
- Filter
- Cuvette
- Detector
- Signal processing electronics

1.7 Dynamic Light Scattering (DLS)

DLS is a non-destructive technique for measuring the size or size distribution of molecules and particles. The principle is based on the scattering effect of particles or molecules that are irradiated by a LASER light. The scattering can trigger constructive or destructive interferences and the resulting light intensity changes can be detected as a random diffraction pattern – also called a speckle pattern. Due to the fact that the particles in a suspension move in BROWNIAN motion, their positions change. These movements lead to fluctations in the speckle pattern. Hence information on the motions of the particles is encoded in the random signal ([36]).
To determine the size of particles in a dispersion, the diffusion constants of the particles are measured and by using the STOKES-EINSTEIN equation (see eq. 8) the particle radius can be calculated ([36]).

$$D_0 = \frac{k_B T}{6 \pi \eta r} \tag{8}$$

For a description of D_0, k_B, T, π, η and r see "List of Symbols" in the Appendices.

The following parts are considered as basic equipment that is needed for DLS apparatus:

- Light source
- Optics
- Cuvette
- Detector
- Signal processing electronics

Due to the device performance of the DLS apparatus used, only average particle diameters between a range of $0{,}3\,\text{nm} - 10\,\mu\text{m}$ can be measured and a minimum sample volume of $12\,\mu\text{L}$ is needed.

1.8 Background

As already mentioned, an overall aim of this thesis is to gain further knowledge or maybe disclose the secret of the Tailing. For this purpose, the disinfected *MS2* and the belonging host *E. coli* were used along with the disinfectant Chlorine Dioxide.
To shed light on the phenomenon, different experiments have been performed to verify three different hypotheses. Therefore already proposed hypotheses ([22]) have been adopted.

Hypothesis 1 — Loss of biocidal properties of the experimental solution

The idea behind this hypothesis is that an altered disinfecting curve is a result of a changing environment during the experiment. Because of this, a loss of the biocidal properties of the disinfectant occurs and leads to the observed Tailing phenomenon. The longer the experiment lasts, the less effective the disinfection process becomes.
The different parts of the observed disinfection curve could be explained as follows:

- In the beginning, the disinfection process behaves as expected. This results in a sharp decrease of the phage population.
  $\Longrightarrow$ (Nearly) Linear range of the disinfection curve
- A distinct time period later, the change in the biocidal properties of the disinfectant leads to a continously declining killing rate.
  $\Longrightarrow$ Transition range of the disinfection curve
- After further experimental duration, a complete loss or – at least a nearly complete loss – of the biocidal properties of the disinfectant results in a nearly stable viability of the remaining phages. This can be observed as the number of dead phages approaches zero.
  $\Longrightarrow$ Tailing range of the disinfection curve
- Any extension of the experiment implies virtually no further decimation.
  $\Longrightarrow$ Ongoing Tailing range

Hypothesis 2 — Aggregation of virus particles

Another explanation that attributes the altered disinfection curve to occuring aggregation between virus particles.
An aggregate usually leads to minimized contact surface for each particle compared to its non-aggregating form. But this do differ significantly depending on the position of the particles in an aggregate. Particles on the outside will have clearly increased contact surface to the environment, whereas particles on the inside have decreased contact surface. That is due to the structure of an aggregate, which also explains the different reactions that arise from the changing conditions of the close environment.
Based on this hypothesis, the obtained disinfection curve can be explained as following:

- The first contact between phage particles and the disinfectant results in many dead phages.
  $\Longrightarrow$ Nearly linear range of the disinfection curve
- The longer the experiment lasts, the more external attached particles are killed. However, with each dead particle less are available for the disinfectant, leading to a reduced disinfection rate.
  $\Longrightarrow$ Transition range of the disinfection curve
- Adsorption and desorption processes on the surface of the aggregates will either make some protected particles available for further disinfection or reinforce their protecting shield.
  $\Longrightarrow$ Tailing range of the disinfection curve
- The available amount of dead particles increases with time. This occurs to such an extent that there are many potential adsorbing particles available for each desorbed particle, which leads to a shortened exposure time.
  $\Longrightarrow$ Ongoing Tailing range

Hypothesis 3 — Existence of a resistant virus subpopulation[2]

This explanation points in a completely different direction, as it focuses on the present *MS2* population. The important property is an assumed heterogeneity within the population that results in at least two subpopulations that differ significantly in their resistance behavior against the impact of ClO_2. The majority of phage particles (1^{st} subpopulation) possesses only a low resistance capacity, while a minority of phage particles (2^{nd} subpopulation) possesses a high resistance capacity.
The resulting disinfection curve could be explained as following:

- In the first time period, phages of the 1^{st} subpopulation are killed very quickly, leading to a sharp decrease in the subpopulation. Unlike the 1^{st} subpopulation, phages of the 2^{nd} subpopulation are not affected and thereby not decimated.
  $\Longrightarrow$ (Nearly) Linear range of the disinfection curve

- After a period of time, most of the phages of the 1^{st} subpopulation have been killed, and for that reason the decrease decelerates. Phages of the 2^{nd} subpopulation are still not affected – or only barely – and they contribute just a minimum amount to the decrease, as a result.
  $\Longrightarrow$ Transition range of the disinfection curve

- The longer the influence time, the fewer phages of the 1^{st} subpopulation are still alive, and therefore their contribution to the shape of the disinfection curve also decreases. In contratst, a large number of the phages of the 2^{nd} subpopulation are still alive which means that they now contribute to the shape of the disinfection curve to a larger extent.
  $\Longrightarrow$ Tailing range of the disinfection curve

- After further exposure of the phages to the disinfectant, the whole 1^{st} subpopulation is killed. Contrary to the 1^{st} subpopulation, only a very low percentage of the 2^{nd} subpopulation is decimated .
  $\Longrightarrow$ Ongoing Tailing range

[2] A very important precondition for this hypothesis is that the distribution between the two subpopulations is very unequal, with the vast majority in the 1^{st} subpopulation. Otherwise the distinct shape of the observed disinfection curve could not be explained by this hypothesis.

2 Experimental

2.1 Materials

2.1.1 Virus dilution buffer preparation

For the preparation of 1 L Virus dilution buffer (VDB) (5 mmol/L Phosphate (PO_4^{3-}), 10 mmol NaCl, pH 7,5) 0,78 g Sodium dihydrogen phosphate (NaH_2PO_4) and 0,58 g NaCl were filled up with Ultrapure water (Milli-Q water) and the pH became adjusted.

2.1.2 Preparation of Sodium thiosulfate solution

To produce a 10% Sodium thiosulfate ($Na_2S_2O_3$) solution, 50 mg of $Na_2S_2O_3$ was solved in 500 μL of VDB. The solution had to be prepared daily.

2.1.3 Preparation of Lysogeny broth

For 1 L Lysogeny broth (LB) 10 g Tryptone, 1 g Yeast extract and 8 g NaCl were filled up with Milli-Q water.

2.1.4 Overnight *Escherichia coli* solution

15 mL LB were filled into a test tube and 100 μL of thawn *E. coli* was added. Afterwards the test tube was stirred over night at 37 °C. The whole described procedure had to be performed under sterile conditions.

2.2 Methods

2.2.1 Chlorine Dioxide Stock solution production

To produce the ClO_2 stock solution, the following steps were carried out according to Gates (The experimental setup is shown in fig. 5:

1. First a few solutions were prepared
   - Solution 3a: $NaClO_2$ (8 g/100 mL water)
   - Solution 3b: Potassium persulfate ($K_2S_2O_8$) (4 g/100 mL water)
   - Solution 4: $NaClO_2$ (2 g/200 mL water)
2. Next, the sketched bottles in fig. 5 were filled as described:
   - Bottle 1: Empty
   - Bottle 2: Approximately 200 mL Milli-Q water
   - Bottle 3: 100 mL of Solution 3a is carefully mixed with 100 mL of Solution 3b
   - Bottle 4: Approximately 200 mL of Solution 4
   - Bottle 5 (hold on ice): Approx. 600 mL Milli-Q water
3. Afterwards Nitrogen (N_2) was turned on until gentle bubbling could be observed
4. As soon as the solution in bottle 5 had changed colour into an intensive yellow, the N_2 was turned off.
5. The achieved ClO_2 solution in bottle 5 was divided into several light protected, brown-coloured glass bottles.
6. The concentration of the ClO_2 solution was determined by spectrophotometric measurement at 358 nm.

2.2.2 Propagation and purification of *Enterobacteria phage MS2*

The *MS2* purification had been performed in accordance with Pecson et al..

Day 1 (preparations) First of all, the media had to be manufactured. Therefore 2 L of LB were prepared, equally portioned into two bottles (1 L each), and autoclaved for 20 min at 121 °C and 1 bar excess pressure. After autoclavation, 100 mL Calcium Chloride ($CaCl_2$) solution with D-Glucose and 1 mL of Streptomycin was added to each LB containing bottle.
In addition, an *E. coli* overnight culture was prepared and grown at 37 °C and flasks were tempered overnight at 37 °C.
All described processes of day 1 were performed under sterile conditions.

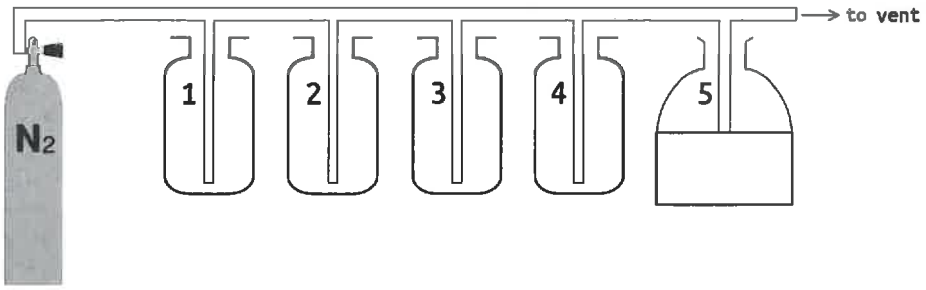


Figure 5: Experimental setup for the Chlorine Dioxide stock solution production.
(All sketched materials were obtained from LLC under the license of CC 0 1.0)

Day 2 Before adding 5 mL of the overnight *E. coli* culture to each LB flask, a blank for the spectrophotometric measurements had been collected.
Therefore 5 mL of one LB flask was removed and afterwards the propagation of *E. coli* was started by shaking both flasks with 80 rpm at 37 °C in a shaking incubator. Through continuous sampling and spectrophotometric measurement of the Absorbance at 600 nm, the growth status of the *E. coli* could be determined (see table 1), whereas the already taken pure LB sample served as blank. A minimum Absorbance of 0,04 had to be reached before the 10 μL stored *MS2* stock was added into each flasks. As shown in table 1, the *MS2* Stock was added after 70 min of cultivation time.

Table 1: Measured Absorbance during the *Enterobacteria phage MS2* propagation and purification process

Cultivation time / min	Flask	Absorbance
30	1	0,02
	2	0,02
70	1	0,06
	2	0,06

To enable the infection process each bottle was left for approximately 5 h without shaking[3]. The processes of day 2 described so far were performed under sterile conditions.
After the resting time, 5 mL of $CHCl_3$ were added to each bottle and both were shaken for at least 15 min to ensure a successful lysis of the bacteria and release of any virus. To finishing the process, all $CHCl_3$ was removed and an additional 10 min of air bubbling through each bottle was performed to expel any residual $CHCl_3$.
A 1 mL sample for plating was collected.
Both solutions were separated into 250 mL bottles, each of them was centrifugated for 15 min at $4000 \times \vec{g}$, and the superantant of all were unified in two bottles each containing 100 g of Polyethylene glycol (PEG) 6000 and 29,22 g of NaCl.
After filling up both to 1 L with Milli-Q water, the PEG and NaCl were dissolved by shaking and the flask was left overnight at 4 °C.

Day 3 First both flasks were centrifugated for 45 min and $7000 \times \vec{g}$.
After removing and rejecting the supernatant, approximately 5 mL of VDB were added to the pellet without resuspending and was left for at least 2 h to soak. The remaining solution of each bottle became unified, whereas approximately 5 mL of VDB were used to rinse out all bottles one after the other to save possible remains.
In the next step, the unified liquids became portioned into 2 mL tubes and all of them were centrifuged for 7 min at $10000 \times \vec{g}$. The resulting supernatant of each tube was transfered into a new tube, and 10 mL of $CHCl_3$ were added. After mixing and centrifuging again for 10 min at $3000 \times \vec{g}$, each aqueous phase (upper phase) was transferred into a new tube. By air bubbling through both bottles for 10 min any residual $CHCl_3$ was expelled and the gained $CHCl_3$ free virus suspension were concentrated in two Ultra centrifugal filter devices by centrifuging for 15 min at $3000 \times \vec{g}$.
The remaining suspension was washed with 5 mL of VDB three times, collected and sterile filtered so that in

[3]Due to the fact that *MS2* infects *E. coli* via entry through the F-Pilus, shaking could prevent the proper building of F-Pili

the end two different virus stock solutions, with a final volume of almost 1 mL each, were received.
To determine the concentration of the two produced stock solutions a Double-layer plaque assay experiment was performed.

2.2.3 Sterile filtration

The whole process was carried out under sterile conditions. Before starting the real filtration process, a few preparations had to be done.
First a syringe that had been connected with a needle was rinsed with VDB. In the next step, a syringe filter was added to the already attached parts, which resulted in a complete syringe unit (syringe + syringe filter + needle). In addition, the syringe filter was moistened with VDB. Then the virus filtration could be carried out.

1. First the syringe filter was removed so that the virus solution could be drawn.
2. This was followed by the replacement of the needle by the syringe filter.
3. The virus solution became sterile filtered by pressing it through the syringe filter, where the first 2 drops were rejected (remaining VDB in the syringe filter)[4].

2.2.4 Standard disinfection experiment

Preparation of the Chlorine Dioxide solutions

For this purpose, approximately 100 mL of VDB were transferred into a brown coloured bottle and a defined volume of ClO_2 stock solution was added. To receive the required concentration, it was necessary to alternate between adding ClO_2 stock solution and spectrophotometric measurement at 358 nm. In the end, two solutions with different concentrations and their respective Absorbance were created:

- A submitting solution with a concentration around 0.5 mg/L ($\widehat{=}$ $Abs_{358\,nm} = 0.009$).
  $\Longrightarrow$ Solution 1
- An adding solution with an Absorbance around 0.28 ($\widehat{=}$ 16 mg/L).
  $\Longrightarrow$ Solution 2

During a disinfection experiment, the concentrations have to stay constant or at most vary in a minimal range. This is ensured by solution 2 and an automatic dosing unit that squeezes an attached syringe – connected with needle and a hose – with a constant velocity.
Before installing the syringe, it had to be rinsed and filled with approximately 10 mL of Solution 2. After connecting the needle with the syringe, a hose was put on the needle. Afterwards, the dosing unit was snapped into the starting position and switched on for a few seconds to rinse the whole system (syringe + needle + hose).

Pre-disinfection run

1. Before starting an experiment, a beaker was rinsed with 5 mL of Solution 1 (Submitting solution).
2. Then 4 mL of Solution 1 were placed into the beaker and the timer was switched on.
3. After 30 s a sample of 1 mL had to be taken and the Absorbance was measured.
   $\Longrightarrow$ It should had stayed at more or less a constant value compared to the starting concentration
4. Once the concentration stayed constant, the stirrer was switched on and Solution 2 (Adding solution) was added.
5. After the pre-disinfection run was performed, a 1 mL sample of the solution had to be taken and the Absorbance was measured. The concentration should have stayed constant during the whole experiment.
   $\Longrightarrow$ ClO_2 concentration **Before** (see table 3)

[4] To collect the remaining sterile virus solution, the VDB was drawn up again (only with the needle and syringe). After replacing the needle again with the syringe filter, the first 2 drops of the solution were collected.

Disinfection run

1. The real disinfection began with adding virus into the experimental beaker (For the added volumes and the corresponding virus concentration see table 4).
2. At each defined time point (see table 2) a sample of 100 μL was taken and immediately mixed with 5 μL of $Na_2S_2O_3$[5].
3. After the last sample the Absorbance of the solution was measured again.
   $\Longrightarrow$ ClO_2 concentration **After** (see table 3)

Table 2: Typical time points of sampling during a standard disinfection experiment

Sample	Time /s
1	0
2	10
3	30
4	60
5	90
6	150
7	210
8	300

2.2.5 Chlorine Dioxide concentration determination

To determine the ClO_2 concentration Photometry has been used. Around 1 mL of the sample was filled into a cuvette and placed in the cuvette holder. Whereby the direction mark on the cuvette always faced the site of the incident light. The photometer was closed and the Absorbance at 358 nm measured. Based on the received Absorbance, the according ClO_2 concentration was calculated using eq. 9.

$$\frac{A_{358\,nm} * M(ClO_2) * Conversion\ Factor}{\varepsilon} \tag{9}$$

The calculation is also shown by an example with a measured Absorbance of 0,0098 (see eq. 10).

$$\frac{0.0098 * 67.45 * 1000}{1250} = 0.53\,\mathrm{mg\,L^{-1}} \tag{10}$$

For a description of A, M and ε see "List of Symbols" in the Appendices.

2.2.6 Variations of the standard experiment

Experiment 1.1

Readding of the same volume of virus solution to the experiment after 120 s.

Experiment 1.2

Adding ClO_2^- in form of $NaClO_2$ immediately before starting the experiment.
Whereas two different amounts of ClO_2^- had been added. Once half of the according ClO_2^- dose that is built during the experiment and once the equal ClO_2^- dose. That meant 7 μL of a $NaClO_2$ solution (Concentration of approximately 10^{-2}mg/μL) for the half dose and 14 μL of the same $NaClO_2$ solution for the equal dose was added.

[5]The $Na_2S_2O_3$ is necessary to quench the ClO_2 and thus stop the disinfection process immediately after sampling.

Table 3: ClO_2 concentrations of the performed experiments

Experiment	Run	Absorbance		Concentration /(mg/L)		
		Before	After	Before	After	Difference
0.1	1	0,0080	0,0071	0,43	0,38	0,05
	2	0,0068	0,0088	0,37	0,47	0,10
	Stock AA	0,0076	0,0076	0,41	0,41	0
	Stock AB	0,0076	0,0076	0,41	0,41	0
0.2	-	0,0112	0,126	0,60	0,68	0,08
1.1	1	0,0091	0,0084	0,49	0,45	0,04
	2	0,0098	0,0098	0,53	0,53	0
1.2	Full dose	0,0062	0,0062	0,33	0,33	0
	Half dose	0,0065	0,0055	0,35	0,30	0,05
2.1 & 2.2	Standard	0,0112	0,0121	0,60	0,65	0,05
	Filtered	0,0104	0,0132	0,56	0,71	0,15
	Sonicated	0,0089	0,0125	0,48	0,67	0,19
2.3 & 2.4	Standard	0,0107	0,0114	0,58	0,62	0,04
	Concentrated VDB	0,0099	0,0105	0,53	0,57	0,04
	$CHCl_3$ extracted	0,0102	0,0085	0,55	0,46	0,09
2.5	Standard with pause	0,0102	0,0139	0,55	0,75	0,20
	Sonication	0,0087	0,0207	0,47	1,12	0,65
2.6	10^8 pfu/mL	0,0074	0,0081	0,40	0,44	0,04
	10^{10} pfu/mL	0,0060	0,0083	0,32	0,45	0,13
3.1	1	0,0082	-	0,44	-	-
		0,0097	0,0117	0,52	0,63	0,11
	2	0,0094	-	0,51	-	-
		0,0094	0,0099	0,51	0,53	0,02
	3 (After)	0,0102	-	0,55	-	-
		0,0089	0,0089	0,48	0,48	0
3.2	Standard	0,0090	0,0075	0,49	0,40	0,09
	Changing solution	0,0103	0,0488	0,56	2,63	2,07

Experiment 2.1

The added virus solution became filtrated before starting the disinfection experiment via sterile filtration (For the filtration procedure see 2.2.3).

Experiment 2.2

The added virus solution was sonicated for 30 min before starting the disinfection experiment.

Experiment 2.3

The used virus solution became $CHCl_3$ extracted before the experiment.Therefore 200 μL of $CHCl_3$ and 200 μL of virus dilution (1:10) was transferred into a micro tube and became shaken.

Experiment 2.4

A VDB with a NaCl concentration (c = 500 mmol/L) in the experimental solution fifty times higher than the concentration in the standard experiment was used. Therefore 4,26 g NaCl was added to 150 mL VDB. Afterwards the buffer became sterile filtrated.

Experiment 2.5

The experimental solution became sonicated after 120 s for 10 min. Afterwards the disinfection experiment started again.

Experiment 2.6

Experiments with lower virus concentration were performed on ice. One experiment included a starting virus concentration of approximately 10^8pfu/mL and a second included a starting virus concentration of approximately 10^{10}pfu/mL. The ice was necessary to slow down the disinfection rate, since at RT such experiments would not be possible due to the reached disinfection rate.

Experiment 3.1

To get rid of the $Na_2S_2O_3$, the experiment was quenched after 120 s and five washing steps were applied. (For the washing procedure see 2.2.7). Afterwards, the experiment restarted.

Experiment 3.2

An experiment was performed with an increased concentration of the added solution. Therefore a five times higher concentrated Solution 2 (adding solution) was used.

Table 4: Approximately virus concentrations in the experimental solution during the performed experiments.
Note: The total volume is the same for all experiments ($V_{tot} = 2$ mL)

Experiment	Name	Virus stock Titer / (pfu/mL)	Dilution	Volume / μL	Concentration / (pfu/mL)
0.1	A	$8{,}7 \times 10^{12}$	1: 10	40	ca. 2×10^{10}
0.2	AB	$7{,}8 \times 10^{14}$	1: 10	160	ca. 6×10^{12}
1.1	A	$8{,}7 \times 10^{12}$	1:10	120	ca. 5×10^{10}
1.2	A	$8{,}7 \times 10^{12}$	1:10	40	ca. 2×10^{10}
2.1	AB	$7{,}8 \times 10^{14}$	1:100	160	ca. 6×10^{11}
2.2	AB	$7{,}8 \times 10^{14}$	1:10	160	ca. 6×10^{12}
2.3	AB	$7{,}8 \times 10^{14}$	1:10	80	ca. 3×10^{12}
2.4	AB	$7{,}8 \times 10^{14}$	1:10	160	ca. 6×10^{12}
2.5	AB	$7{,}8 \times 10^{14}$	1:10	160	ca. 6×10^{12}
2.6	AB	$7{,}8 \times 10^{14}$	1:1000	160	ca. 6×10^{10}
	AB	$7{,}8 \times 10^{14}$	1:100000	160	ca. 6×10^{8}
3.1	AB	$7{,}8 \times 10^{14}$	1:10	160	ca. 6×10^{12}
3.2	AB	$7{,}8 \times 10^{14}$	1:10	160	ca. 6×10^{12}

2.2.7 Washing procedure

To determine the number of washing steps needed to get rid of the $Na_2S_2O_3$ that was added to quench the ClO_2, a 1^{st} pre-experiment was performed as described.
Therefore a test solution was produced, composing of 400 μL VDB, 5 μL $Na_2S_2O_3$, 100 μL ClO_2 solution but without any virus dilution.

Washing step 1
400 μL of the test solution were transferred into a centrifugal filter tube and the whole filter unit (centrifugal filter + tube was centrifugated for 3 min at 8000 rpm afterwards the filtrate became rejected.

Washing step 2
400 μL VDB had been added into the centrifugal filter tube and once again centrifugated at the same conditions as before (3 min at 8000 rpm and rejecting of the filtrate).

Further washing steps
Repeating of Washing step 2 as long as an alteration of the Absorbance could be detected.

After each washing step, the following procedure was performed before measuring the Absorbance at 358 nm:

1. Removing the centrifugal filter and putting it into a new tube in a reversed position (bottom-up).
2. Centrifugating for 1 min at 2000 rpm.
3. To increase the volume and transfer the retentate into a tube, 50 μL VDB had to be added.

4. Adding 1 mL of Solution 1.

The measured Absorbance of the pre-experiments can be seen in table 5.

Table 5: 1^{st} pre-experiment to determine the number of necessary washing steps to get rid of the quenching reagent

Washing step	Absorbance
0	0,0014
1	0,0013
2	0,0010
3	0,0025
4	0,0101
5	0,0126

A 2^{nd} pre-experiment was performed under exactly the same conditions, but with 5 μL virus dilution (1:10) in the test solution. The measured Absorbance can be seen in table 6 .

Table 6: 2^{nd} pre-experiment to determine the number of necessary washing steps

Washing step	Absorbance
0	0,0008
1	0,0006
2	0,0003
3	0,0032
4	0,0055
5	0,0055

In both experiments, the Absorbance increased after the fourth washing step[6]. To be sure that most of the remaining $Na_2S_2O_3$ is removed, five washing steps had been chosen as the appropiate number. Therefore for all further experiments in which a washing procedure was necessary, five washing steps are performed. But before starting such an experiment, further treatment is required in order to use the whole experimental solution that is available. Therefore the following additional concentration step was introduced:

Concentration step

The whole experimental solution became transferred into a centrifugal filter in portions of 400 μL and the whole filter unit (centrifugal filter + tube) was centrifuged for 3 min and 8000 rpm each time.

2.2.8 Preparing dilutions

All performed dilutions were carried out in Micro test tubes.
To estimate the volume ratio that was needed to end up with the desired dilution, the following considerations were applied. A dilution can be seen as a ratio between one part and the sum of all parts. More precisely, the numerator determines the number of parts of one object whereas the denominator determines the whole.
The maximum dilution step was 1:100.

To show the executed procedure, the 1:100 dilution provides a pertinent example.

1. $\frac{1}{100}$ stands for 1 part (numerator) in 100 parts (denominator)
2. That could be achieved by combining 1 part of something with 99 parts of something else
   $\Longrightarrow 1 + 99 = 100$
3. In our case a part stands for a distinct volume
4. That means for 1 mL in total, 10 μL virus solution had to became mixed with 990 μL VDB
   $\Longrightarrow 10 + 990 = 1000 \equiv 1 + 99 = 100$

The two mainly used dilutions steps were the 1:10 and the **1:100 dilution.**

[6]The differences of the exact values are due to the concentrations of the used ClO_2 solution. This was not relevant for the pre-experiment, as the aim had been to detect just a distinctive increase of the Absorbance.

2.2.9 Double-layer plaque assay

The method was performed following an EN ISO standard ([24]). For the whole plating procedure, it was important to work under aseptic settings.
For each plate, the following steps had been maintained:

1. First, 200 μL of overnight *E. coli* culture were transfered into the Top-Agar containing tube[7].
2. Second, the appropriate dilution of 100 μL was shaken and pipetted into the same tube.
3. Third, the whole test tube became agitated and plated on Petri-dishes containing the Bottom-Agar[8].
4. After 20 min, the agar hardened and the dishes could be incubated in an inverse position ("bottom-up") overnight at 37 °C.

2.2.10 Determing the number of plaques

To guarantee a constant quality of the results, the counting had to always be performed under the same conditions.
Therefore some counting rules had to be respected (see Counting rules). The counting procedure consisted of three major steps. First, the upper lid of the Petri-dish was removed. Second, the remaining lower lid that contains the plated dilutions was held up to the Light source (see Light source) at a distance of about 20 cm. Third, each observed plaque was counted in accordance with the Counting rules.
In the end, each plate possessed a distinct number of plaques, and in consideration of the dilutions and the plated aliquot, the according pfu value could be determined.

Light source

An ordinary desk lamp served as light source. Sunlight would have been also a possibility, but due to the changing of the light properties over the course of the day, misinterpretations could happen more easily. For a better contrast, two blue coloured transparent covers were attached onto the lampshade so that the whole transmitted light had to pass through the transparent covers.

Counting rules

1. The plates have to be free of contaminations as well as the *E. coli* controls.
2. The media composition has to be in a suitable condition.
3. *E. coli* has to be spread homogenously over the whole plate.
4. Only plates with less than 300 pfu are counted.
5. Each observed plaque is counted and marked with a dot.
6. A plaque is not counted if it spread beyond the fringe area of the dish.
7. If there are a lot of plaques on a single plate, the plate can be halved or quartered. Then only one half or quarter needs to be counted and the result could be extrapolated to determine the number of plaques on the full plate.
8. In the case that plaques have grown together as so called twins, they have to be treated as a single plaque.

2.2.11 Determination of plaque forming units

The counted plaques per dish became related to 1 mL for comparability reasons. Therefore all dilution steps and aliquots have to be taken into consideration. This leads to eq. 11. In eq. 12 an example – dilution of ($1:10^9$), 40 plaques counted, 100 μL aliquot – is given.

$$\frac{Plaques \cdot Dilution}{Aliquot} = pfu/mL \tag{11}$$

[7] Before use, the Top-Agar was melted in a water bath at 95 °C for approximately 10 min. Afterwards the tube was tempered at 56 °C.
[8] To spread out the solution equally each dish was moved like a lemniscate.

$$\frac{40 \cdot 10^9}{0{,}1} = 4 \times 10^{11}\, pfu/mL \tag{12}$$

For a description of pfu see "List of Abbreviations" in the Appendices.

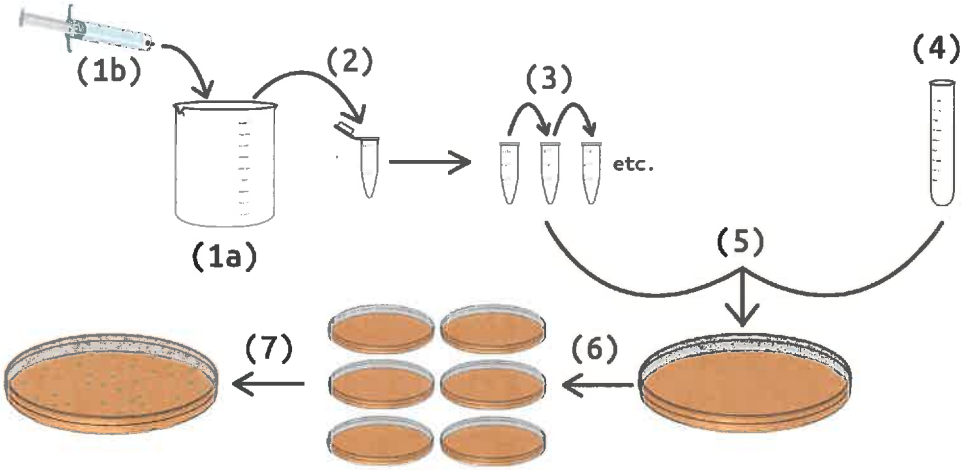


Figure 6: Summary sketch of all critical steps in the cause of a characteristic experiment. To a gently stirred experimental beaker (1a), filled with Solution 1 and Virus Stock solution, Solution 2 is added continuously (1b). Samples are taken at distinct timepoints (2) and dilutions are prepared (3). An overnight *E. coli* solution (4) and the dilutions were transferred into tubes containing Top-Agar and plated on Petri-dishes containing Bottom-Agar (5). The dishes become incubated overnight (6) and finally the resulting plaques are counted (7).
(All sketched materials were obtained from LLC under the license of CC 0 1.0)

2.2.12 Dynamic Light Scattering (DLS)

Measurements were performed in the Zetasizer DLS device. Therefore UV-cuvettes were used and always placed in the same orientation into the cuvette holder. The sample volume of the cuvette was 70 µL and special care was taken to avoid possible air bubbles in the cuvettes that would falsify the results. The temperature was at a constant value of 22 °C during all performed measurements.

DLS pre-experiments

A few pre-experiments, which where based on a basic pre-experiment, were performed. For that basic pre-experiment, the solution that had been measured was composed of 180 µL of VDB and 20 µL of a virus dilution (1:10). Additional further runs with variations of the basic pre-experiment were also performed. More precisely, the solution properties became altered or physically treated as following:

- pH: Measurements were carried out at different pH values of the experimental solution – 180 µL VDB and 20 µL virus dilution (1:10).
  - pH 9,0
  - pH 7,6
  - pH 3,1
  - pH 1,9
- Salt concentration: Measurements of experimental solution – 135 µL VDB and 15 µL virus dilution (1:10) – with different NaCl concentration of the VDB were performed.
  - 10 mmol/L (common VDB)
  - 100 mmol/L (received by adding 76,4 mg NaCl to 15 mL of VDB)

- 500 mmol/L (received by adding 434,2 mg NaCl to 15 mL of VDB)

- Sonication: The whole experimental solution – 180 μL VDB and 20 μL virus dilution (1 : 10) – became sonicated before it was measured for two different time periods.
  - 10 min
  - 30 min

- $CHCl_3$ extraction[9]: 150 μL of $CHCl_3$ and 150 μL of virus dilution (1:100) were mixed.

The used volumes of VDB and virus dilution in all experiments were chosen to preserve a virus concentration of approximately 10^{12} pfu/mL, as can be seen in table 7.

Table 7: Approximately virus concentrations in the experimental solution of the DLS measurements.
Note: For all experiments virus stock AB was used (c = 7,8 × 10^{14} pfu/mL)

Experiment	Virus stock dilution	Volume / μL Virus	Total	Concentration (pfu/mL)
Standard	1:10	20	200	ca. 8×10^{12}
pH	1:10	20	200	ca. 8×10^{12}
Salt	1:10	15	150	ca. 8×10^{12}
Sonication	1:10	20	200	ca. 8×10^{12}
$CHCl_3$ extraction	1:100	150	300	ca. 4×10^{12}

Dynamic Light Scattering measurments during a disinfection experiment

Additional DLS measurements were carried out in the course of a washing disinfection experiment, as described in 2.2.7 Washing procedure. With it, samples were taken and measured as followed:

- Before starting the disinfection experiment (Sample BS)
- Before adding $Na_2S_2O_3$ to quench the ClO_2 (Sample BQ)
- After adding $Na_2S_2O_3$ to quench the ClO_2 (Sample AQ)
- After the washing procedure (Sample AW)

For Sample BS, 70 μL of a solution that was composed of 90 μL VDB, 5 μL $Na_2S_2O_3$ and 10 μL virus dilution (1:10) was used.

2.3 Contaminations

Contaminations occured during the whole thesis, leading to the repetition of several experiments. To minimize, them some precautions have been obeyed:

- Cleaning of surfaces with bleach solution (10%) (working bench, pipettes)
- Wearing of protective clothes (Gloves, coat, safety goggles)
- Changing of gloves before every crucial working step (e.g. buffer preparation, diluting, plating)
- Working under aseptic condition if needed
  - Igniting a burner
  - Working near the flame of a burner
  - Flaming glassware after opening and before closing
  - Changing pipette tips if needed

If despite careful examination contaminations happened, all autoclavable materials were autoclaved and the non autoclavable were disposed and replacements were prepared.

[9]Both extraction phases were measured. Additionally, a standard experiment as described before was performed where a virus dilution (1:100) was used instead of the virus dilution (1:10).

2.4 Experiments with Bacteriophage *fr*

To get a broader understanding of the Tailing phenomenon, disinfection experiments with another bacteriophage were carried out. Due to persistent contamination of the plated samples, these experiments were cancelled.

3 Results

3.1 Virus stock solutions

The concentrations of the two prepared Virus Stock solutions AA and AB can be seen in table 8. Additional Virus Stock solution A (provided by the supervisor) is also included.

Table 8: Concentration of the produced Virus Stock solutions (AA, AB) and a received one (A)

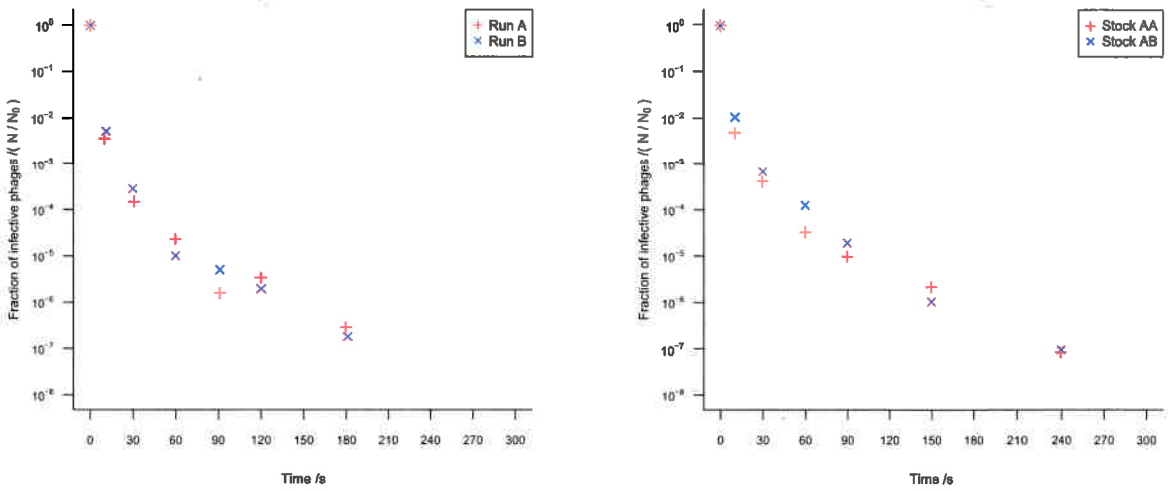
Virus Stock solution	Concentration /(pfu/mL)
A	$8{,}7 \times 10^{12}$
AA	$5{,}7 \times 10^{13}$
AB	$7{,}8 \times 10^{14}$

For all disinfection experiments Virus Stock solution A and AB have been used (Except for the experiments with Bacteriophage *fr*).

3.2 Disinfection experiments

3.2.1 Standard disinfection experiment

Experiment 0.1 In fig. 7, the results of disinfection runs – which were executed as described in the previous section – are plotted. The course of Stock solution A as virus source is shown in graph 7a, whereas in graph 7b, Stock solutions AA and AB were used as virus source. In both graphs, Tailing behaviour can be observed. The Linear range lies within the first 30 s of the experiment followed by the Transition range between 30 s and 150 s. After approximately 150 s, the Tailing range of the disinfection curve is reached.



(a) Virus Stock A

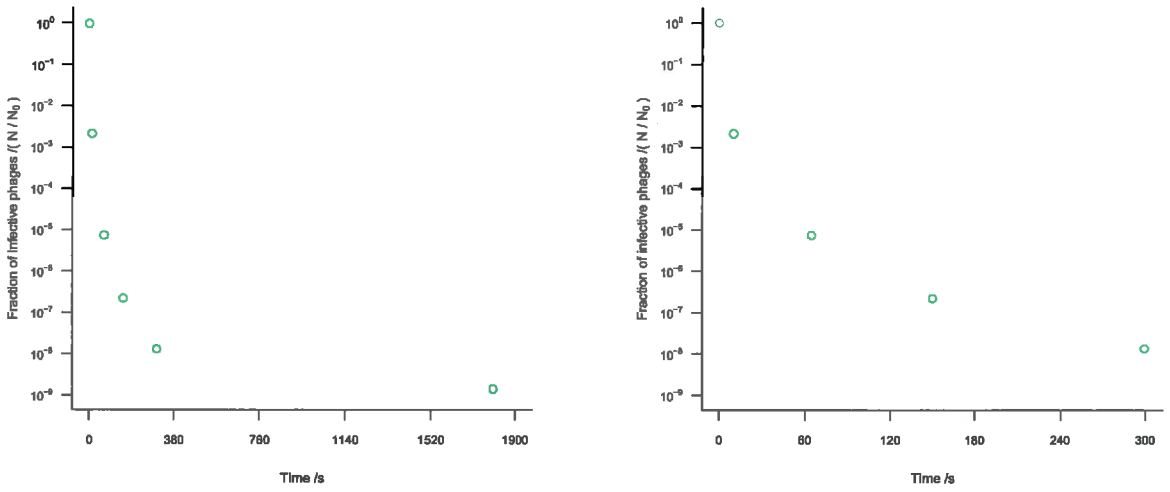
(b) Virus Stock AA and AB

Figure 7: Standard disinfection experiments with Virus Stock A in graph 7a and with Virus Stock AA and AB in graph 7b. The disinfection curves show the characteristic Tailing behaviour with all three included parts. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

3.2.2 Extended experiment duration

Experiment 0.2 To take a closer look into the further progress of the disinfection course, a longer lasting experiment has been performed. The disinfection curve shows the typical Tailing behaviour (see graph 8a). The Tailing range is especially emphasized due to the extended time course. As shown in the graph, active virus particles can still be found, even after 30 min (1800 s).

In order to better compare the results to those of the standard experiment (see fig. 7), the first 300 s of the run are plotted in graph 8b. The Linear range can be seen during the first 30 s, followed by a flattening between 30 s and 150 s – the Transition range. Afterwards, the curve turns into the Tailing range whereby the course gradually becomes asymptotic to the x-axis.



(a) Complete run

(b) Detailed view of the first 300 s

Figure 8: Standard experiment with extended time course. In 8a the complete experiment is plotted, whereas in 8b a detailed view of the first 300 s of the experiment is shown. The disinfection curve of the longer lasting experiment shows also the characteristic Tailing behaviour with all three included parts. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

In addition to the course of the disinfection, the according ClO_2 concentrations are shown in table 9. These values were received by performing a parallel run. No samples were taken during this run, but the ClO_2 concentration has been measured at distinct points in time that are listed in table 9. The range of the determined concentrations lies between 0,53 mg/L and 0,65 mg/L. Thus, there was only a 0,12 mg/L difference during the 1830 s of the whole experiment, and therefore the ClO_2 concentration can be seen as constant.

Table 9: Course of the ClO_2 concentration during Experiment 0.2

Time /s	Adding rate /(µL/min)	Absorbance	Concentration /(mg/L)
40	12	0,0098	0,53
120		0,0112	0,60
180		0,0106	0,57
240		0,0094	0,51
330		0,0097	0,52
480		0,0099	0,53
630		0,0102	0,55
1080		0,0115	0,62
1200		0,0114	0,62
1500	10	0,0108	0,58
1830		0,0108	0,58

3.3 Hypothesis 1 — Loss of biocidal properties of the experimental solution

3.3.1 Additional Doses of *Enterobacteria phage MS2*

Experiment 1.1 In fig. 9, the results of the experiment are shown. Before additional *MS2* is added to the solution, the disinfection curve is similar to that of the standard experiment – the Linear range occurs during the first 30 s, followed by the typical Transition range. After adding more *MS2*, a disinfection curve that shows Tailing behaviour with almost the same time durations for the Linear, Transition and Tailing range was recorded.

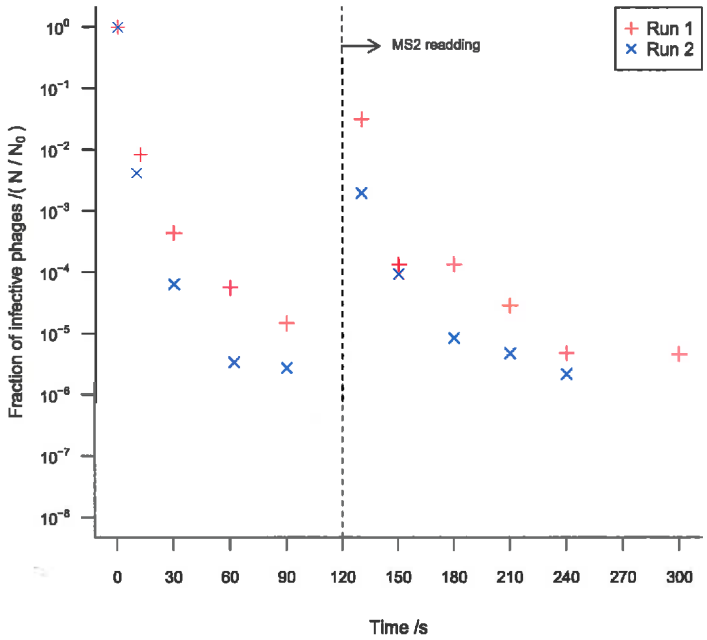


Figure 9: More virus being added to the experimental solution after 120 s. The disinfection curve before adding more *MS2* virus – as well as the further course of the curve afterwards – shows the characteristic Tailing behaviour. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

3.3.2 Adding Chlorite before starting the experiment

Experiment 1.2 In figure 10, the results of the experiment can be seen. The run with the full dose of ClO_2^-, shows the same disinfection course as the other run with the half dose of ClO_2^-. In both runs Tailing behaviour can be seen, including the initial Linear range as well as the characteristic Transition range and the final Tailing range.

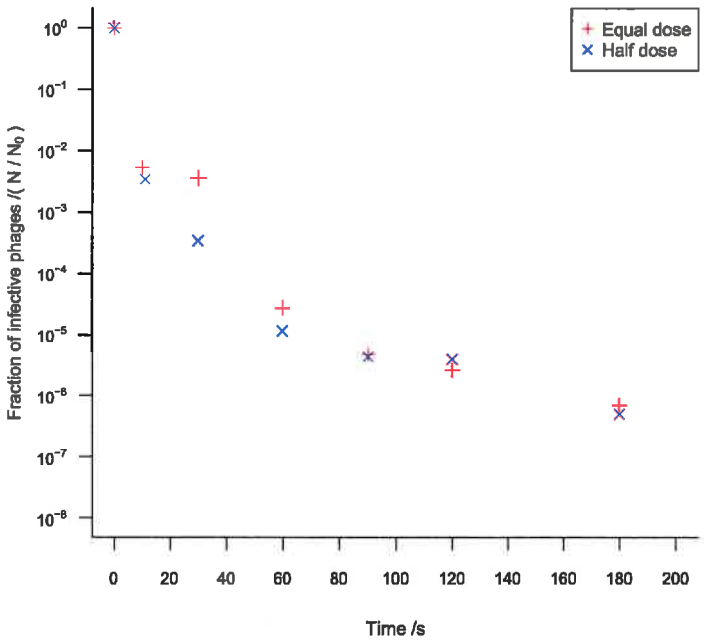


Figure 10: Adding Chlorite before starting the experiment. The disinfection curves of both doses show the characteristic Tailing behaviour with all three included parts. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

3.4 Dynamic Light Scattering measurements

3.4.1 pH variations

In table 10, the results of the DLS measurements of virus containing solutions are shown. For the series 1 and 4, the average particle diameter is 37 nm and 30 nm, respectively. These diameters lie in the range of the theoretical *MS2* diameter (26 nm). Both pH values of the two series (pH 7,6 and pH 9,0) are higher than the pI of *MS2* (pI 3,9). In contrast, the pH in series 2 and 3 are lower than the pI. In series 3, the pH of 3,1 lies very close to the pI. This results in an average particle diameter of 2129 nm, which is about eighty times bigger than the theoretical diameter of a single virus particle. Series 2 at pH 1,9 has an average particle diameter of 373 nm which is nearly fifteen times bigger than the theoretical diameter of a single virus particle.

Table 10: Results of DLS measurements of virus containing solutions at different pH

Series	Run	pH	Average particle diameter /nm per run	per series
1	1	7,6	44,64	37
	2		43,35	
	3		28,27	
	4		28,82	
	5		37,5	
2	1	3,1	2147	2129
	2		2110	
3	1	1,9	379,9	373
	2		366,1	
4	1	9,0	29,57	30
	2		30,72	

3.4.2 Sonication treatment

The results of the DLS measurements after sonication of series 5 and 6 can be seen in table 11. The resulting average particle diameter after 10 min sonication treatment (series 5) is 32 nm and lies in the range of the theoretical diameter of a single virus particle. The same appears for series 6 with a 30 min sonication treatment that results in an average particle diameter of 29 nm.

Table 11: Results of DLS measurements of virus containing solutions after sonication treatment

Series	Run	Sonication duration /min	Average particle diameter /nm per run	per series
5	1	10	31,33	32
	2		31,88	
6	1	30	28,67	29
	2		29,14	

3.4.3 Variations of the NaCl concentration in the VDB

For all three measured series with 10 mmol/L, 100 mmol/L and 500 mmol/L NaCl, respectively, in the VDB, the results are all in the range of the theoretical diameter of a single virus particle (see table 12). For series 8 the average particle diameter is 29 nm, for series 7 it is nearly the same with 30 nm, and for series 9 it is a little bit higher with 35 nm.

Table 12: Results of DLS measurements of virus containing solutions with different NaCl concentrated VDB

Series	Run	VDB /(mmol/L NaCl)	Average particle diameter /nm per run	per series
7	1	10	29,32	30
	2		29,66	
8	1	100	29,37	29
	2		29,37	
9	1	500	34,84	35
	2		35,18	

3.4.4 Chloroform treatment

In table 13 the results of series 10 are shown. In the first three runs, the virus solution became $CHCl_3$ extracted. To separate the resulting phases, the solution was shaken in run 1 whereas in run 2 and run 3 centrifugation was performed. For the Reference run, the virus solution has not been treated with $CHCl_3$. The average particle diameter of runs 1,2 and 3 is 31 nm and very similar to the result of the Reference run with 29 nm.

Table 13: Results of DLS measurements of $CHCl_3$ extracted virus solutions after separation by shaking or centrifugation

Series	Run	Seperation	Average particle diameter /nm per run	per series	Comments
10	1	10 min shaking	31,33	31	
	2	15 min centrifugation	31,88		Upper phase used
	3	15 min centrifugation	28,67		Lower phase used
	Reference	-	29,09	29	

3.5 Hypothesis 2 — Aggregation of virus particles

3.5.1 Dynamic Light Scattering measurements in the course of a washing disinfection experiment

In table 14 the results of the DLS measurements in the course of a washing disinfection experiment can be seen. Also in this case, the average particle diameter of all samples stays at approximately 29 nm. The exact values are 29 nm for Sample BS that was taken before starting the run, 29 nm for Sample BQ that was taken before quenching the experimental solution, 30 nm for Sample AQ that was taken after quenching the experimental solution, and 29 nm for Sample AW that was taken after washing the experimental solution.
This means that the average particle diameter in the course of a disinfection experiment remains practically unchanged and stays within the range of the theoretical diameter of a single virus particle.

Table 14: Results of DLS measurements of samples taken during a disinfection experiment with a washing pause.
Note: BS ... Before starting, BQ ... Before quenching
AQ ... After quenching, AW ... After washing

Measurement	Sample	Average particle diameter /nm
1	BS	29
2	BQ	29
3	AQ	30
4	AW	29

3.5.2 Filtration of the experimental solution

Experiment 2.1 The results of the experiment can be seen in fig. 11. The run with the filtrated experimental solution shows a similar course as the standard experiment. The Linear range occurs during the first 30 s, followed by the typical Transition range between 30 s and 150 s and ending up in the Tailing range afterwards.

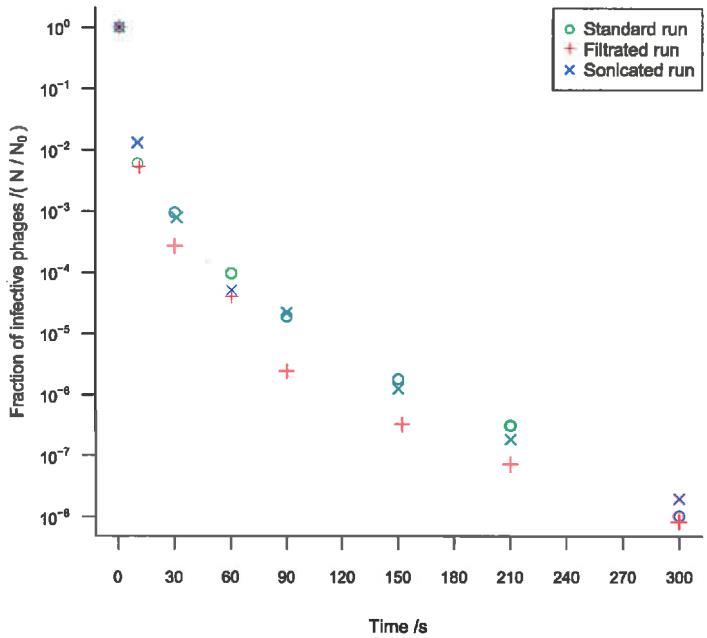


Figure 11: The added virus solution becomes sonicated or filtrated before starting the disinfection experiment. The disinfection curve of the sonicated virus solution – as well as the curve of the filtered virus solution – show the characteristic Tailing behaviour with all three included parts, which can also be seen in the plotted standard experiment. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

3.5.3 Sonication of the experimental solution before starting the experiment

Experiment 2.2 The results of the experiment can be seen in fig. 11. The run in which a sonciated experimental solution is used shows a similar course as the standard experiment. The Linear range occurs during the first 30 s, followed by the typical Transition range between 30 s and 150 s and ending up in the Tailing range afterwards.

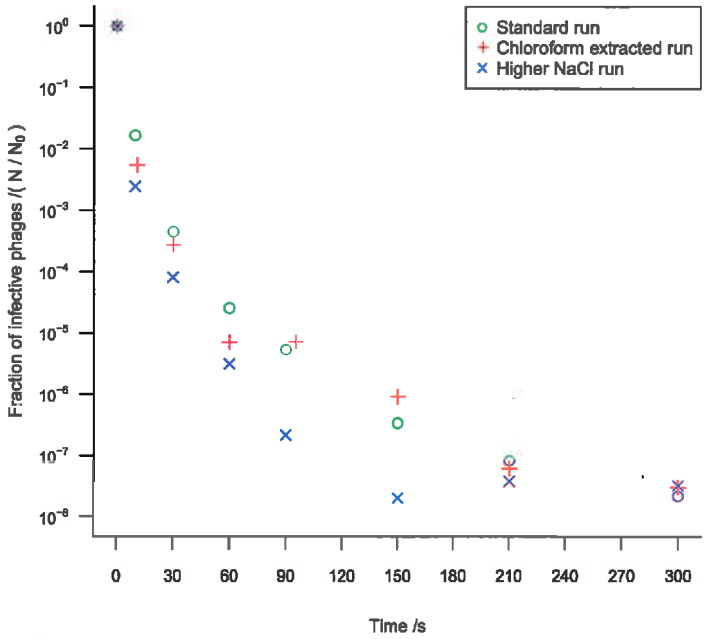


Figure 12: The used Virus Stock solution becomes $CHCl_3$ extracted before the experiment or a higher NaCl concentrated VDB is used. The disinfection curves of the $CHCl_3$ extracted virus solution, as well as the curve of the experiment with higher NaCl concentrated VDB, show the characteristic Tailing behaviour with all three included parts that can also be seen in the standard experiment. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

3.5.4 Chloroform extracted Virus Stock solution

Experiment 2.3 In fig. 12, the results of the experiment can be seen. The run in which the $CHCl_3$ extracted Virus Stock solution is used shows a similar course as the standard experiment. The Linear range occurs during the first 30 s, followed by the typical Transition range between 30 s and 150 s to end up in the Tailing range afterwards.

3.5.5 Higher Chlorine Dioxide concentrated VDB

Experiment 2.4 In fig. 12, the results of the experiment can be seen. The run with a fifty times higher NaCl concentrated VDB in the experimental solution shows a similar course as the standard experiment. The Linear range during the first 30 s, followed by the typical Transition range between 30 s and 150 s to end up in the Tailing range afterwards.

3.5.6 Sonication of the experimental solution after 120 s

Experiment 2.5 In fig. 13 the results of the experiment can be seen. The sonication break – which occur after 120 s for 10 min at a time – result in an ongoing course of the disinfection curve like in the standard experiment. The standard experiment has also been performed with a 10 min break after 120 s. The Linear range occurs during the first 30 s, followed by the typical Transition range between 30 s and 150 s and also the Tailing range after 150 s.

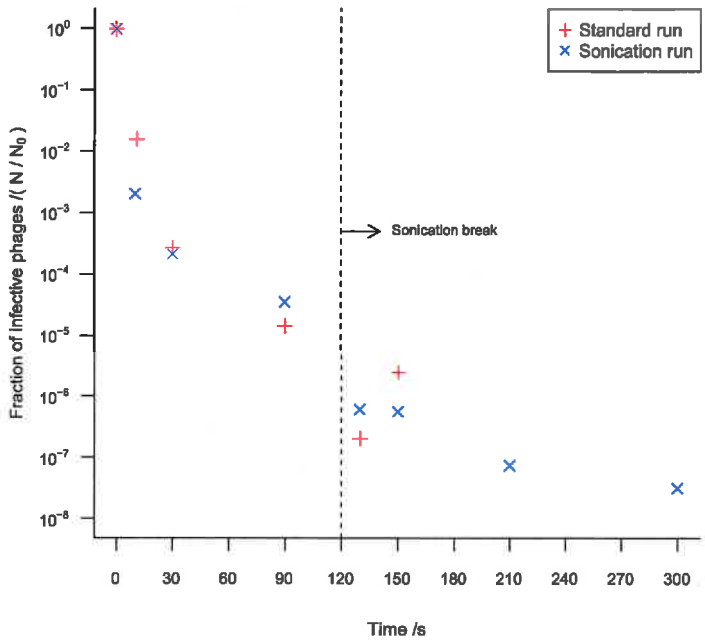


Figure 13: After 120 s both runs have been interrupted for 10 min. The experimental solution of the Sonication run has received sonication treatment during these 10 min, whereas the solution of the Standard run has not been treated. Afterwards, the disinfection process of both runs has been restarted. Both disinfection curves show the characteristic Tailing behaviour, with all three included parts. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

3.5.7 Lower virus concentration on ice

Experiment 2.6 The results of the experiment are shown in fig. 14. Both runs show almost the same Tailing behaviour. However, the chronological sequence of the characteristic ranges is displaced due to a lower experimental temperature because the experiment has been performed on ice. While the Linear range duration is very similar to the standard experiment at around 30 s to 40 s, the Transition range lies explicitly between 60 s to 200 s and the Tailing range starts later as a result.

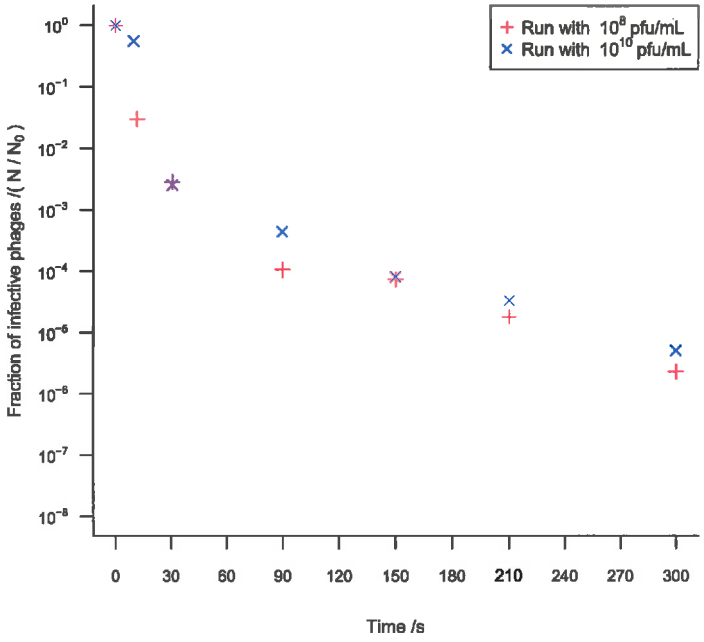


Figure 14: Two disinfection experiments with lower virus concentrations are shown. Both were carried out on ice. The disinfection curves of both experiments show the characteristic Tailing behaviour with all three included parts. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

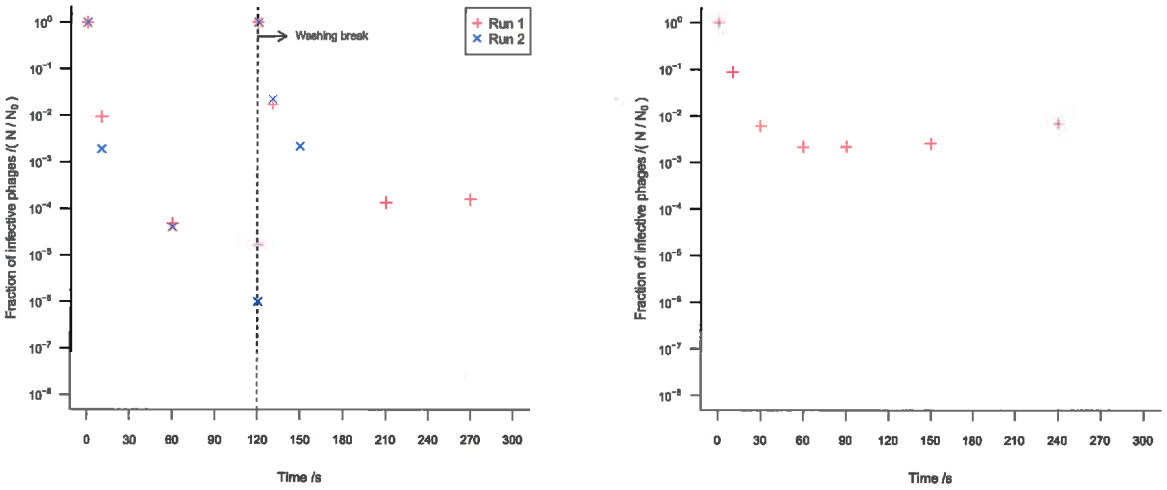
3.6 Hypothesis 3 — Existence of a resistant subpopulation

3.6.1 Washing break during the experiment

Experiment 3.1 The results can be seen in fig. 15. In graph 15a, the courses of two runs with a washing pause after 120 s are shown.
Both show a similar course as the standard experiment before the break. The Linear range during the first 30 s can be found, as well as the typical Transition range between 30 s and 120 s when the runs have been stopped to perform the washing procedure. After restarting, exactly the same courses as before can be observed. For a detailed view of the disinfection curve after the washing pause another run is shown in graph 15b. The rate of disinfection is a little bit lower because the disinfection already performed has lowered the virus concentration.

3.6.2 Changing the concentration of the adding solution

Experiment 3.2 The results of the experiment are shown in fig. 16. The run with the changed adding solution (Solution 2) shows the same typical ranges of a disinfection curve that indicates Tailing before the change. Afterwards, the course of the curve goes on without vast alterations.



(a) Whole disinfection course

(b) Range after washing

Figure 15: In graph 15a, two runs with a washing step after 120 s are plotted. The disinfection curves of both show the characteristic Tailing behaviour with all three included ranges before as well as after the washing break. For a better overall view, the results of an additional run after the washing procedure are shown in graph 15b. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

3.7 Courses of the Chlorine Dioxide concentration during the performed disinfection experiments

The results of the described ClO_2 measurements in the previous section are shown in table 3. Each single run of the performed experiments is named, and the measured absorbance values and the corresponding concentrations are listed.

Experiment 0.1
For all four runs, the concentration is in a range between 0,37 mg/L and 0,47 mg/L.

Experiment 0.2
For the performed run, the measured concentration before starting is 0,60 mg/L and after the run it is 0,68 mg/L.

Experiment 1.1
The concentration of both runs is in a range between 0,45 mg/L and 0,53 mg/L.

Experiment 1.2
During both runs, the measured concentration lies between 0,30 mg/L and 0,35 mg/L.

Experiment 2.1 and 2.2
The three runs that were performed are in a concentration range between 0,48 mg/L and 0,71 mg/L.

Experiment 2.3 and 2.4
The concentration range of the three runs performed lie between 0,46 mg/L and 0,62 mg/L.

Experiment 2.5
The measured concentration after the sonication run reaches 1,12 mg/L. With the beginning concentration of 0,47 mg/L, the lowest value of the experiment occurs in this run.

Experiment 2.6
Both runs lie in a range between 0,32 mg/L and 0,45 mg/L.

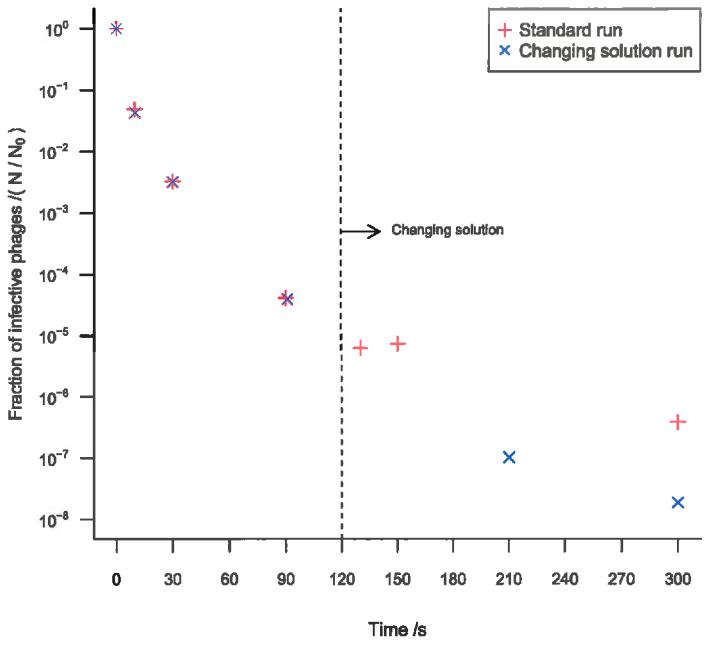


Figure 16: Using a more concentrated adding solution (Solution 2) after 120 s. The disinfection curves of the experiment show the characteristic Tailing behaviour – with all three included parts – before the change to the more concentrated adding solution, as well as after. This can be also seen in the plotted standard experiment before and after the changing event. On the x-axis the time duration is plotted and on the y-axis the corresponding fraction of infective phages is plotted.

Experiment 3.1

For the runs with a washing pause, the detected concentrations are in a range between 0,44 mg/L and 0,63 mg/L.

Experiment 3.2

The concentration of the Standard run lie between 0,40 mg/L and 0,49 mg/L. For the run with an adjusted adding solution (Solution 2) the concentration range is not surprisingly high. Before changing the adding solution, the measured concentration was 0,56 mg/L. Afterwards, it was about 4,7× higher (2,63 mg/L).

4 Discussion

4.1 Standard experiment

Experiment 0.1 Standard experiments were performed to verify that the used virus stock solutions indicate Tailing behaviour. As described in section 3.2.1 Experiment 0.1 all three tested virus stock solutions showed the typical Tailing behaviour.
In each case the characteristic parts that have been described in section 1.2.2 Tailing phenomenon could be found in the resulting disinfection curves. A (nearly) **Linear range** in the beginning followed by a **Transitional range** that is succeeded by the **Tailing range.**

Experiment 0.2 To gain an in-depth knowledge regarding the Tailing phenomenon a disinfection run was performed over a longer period. As already describe in section 3.2.2 Experiment 0.2 the results confirmed that active virus particle remains regardless of the duration. This has also been observed by Hornstra et al..

4.2 Hypothesis 1 — Loss of biocidal properties of the experimental solution

4.2.1 Additional doses of *Enterobacteria phage MS2*

Experiment 1.1 An experiment was conducted to verify a possible alteration of the experimental solution during the inactivation process. Whereby the same volume of *MS2* virus solution has been added again after 120 s. If a loss of biocidal properties of the ClO_2 is the reason for the Tailing behaviour, the additional virus should result in an altered course of the disinfection curve.

As already described in section 3.3.1 Experiment 1.1 that was not the case. The course of the disinfection curves shows the same Tailing behaviour before and after adding of *MS2* virus solution. This observations are in accordance with a similar experiment that Hornstra et al. performed. It seems that a loss of biocidal properties does not occur.

4.2.2 Adding Chlorite before starting the experiment

Experiment 1.2 In this experiment the influence of DBP ClO_2^- in the experimental solution was investigated. First, the half dose of the calculated formed ClO_2^- was added before starting a disinfection run. Secondly, the equal dose of ClO_2^- was added instead. As a DBP ClO_2^- could lead to a change of the solution composition and in this way trigger a loss of biocidal properties of the experimental solution.

The described results of the two previous performed runs in section 2.2.6 Experiment 1.2 do not support this interpretative attempt. The course of the disinfection curves has not been changed due to adding of *MS2* virus solution. No changes in the course of the disinfection curves have been observed in case of adding ClO_2^- before starting a disinfection run, regardless of the added amount. This means a loss of biocidal properties can not be proven.

In consideration of both experiments a loss of biocidal properties as explanation for the Taling phenomenon can be ruled out.

4.3 Hypothesis 2 — Aggregation of virus particles

4.3.1 Dynamic Light Scattering measurements

As hypothesized the existence or formation of aggregates could be the reason for the Tailing phenomenon. Therefore DLS has been used to gain a deeper insight into mean particle diameters in the experimental solution. The existence of aggregates has been studied during several pre-experiments. Whereby not only the average particle diameter in the solution under experimental conditions has been measured, but additionally more extreme conditions have also been applied to get a brighter understanding of the influence of the environment.

The results (see 3.4) of the pre-experiments showed that aggregate formation was favoured if pH < pI. This is in accordance with Langlet et al. as they observed a changement of the diameter from 30 nm at neutral pH to several micrometers when passing the pI.
In view of the pH range of the standard disinfection experiment aggregation, triggerd due to the pH of the environment, it can be excluded as explanation. The reason therefore is that the pH of the experimental solution is obviously higher (pH 7,6) then the pI of *MS2* (pI 3,9).

In the case of sonication treatment, the detected average particle diameter lay in the range of the theoretical diameter of a single virus particle. This is not surprising since Hejkal et al. used sonication to release attached virus particles or to destroy aggregates.
Also with higher NaCl concentrated VDB used, only minimal changes of the average particle diameter have been observed. More precisely, the found average particle diameter in the 500 mmol/L NaCl concentrated solution was only approx. 6 nm higher than in the solution with a conentration of 10 mmol/L NaCl. An increasing of the average particle diameter with increasing concentration is unexpected and contrary to the observations that has been found thus far [37]. However this discrepancy can not be explained by the obtained results. But as these results showed no vast differences of the average particle diameter their influence is not important.
After treatment with $CHCl_3$ the received average particle was in the same range as for the additional performed reference run. This was expected insofar as $CHCl_3$ has been used to disperse virus solutions [38]. Moreover, the techniques used to separate the phases has no influence on the results, neither shaking nor the centrifugation. Besides the organic phase resulting of centrifugation, the average particle diameter of the water phase was shown to be nearly the same diameter. If even in the water phase no aggregates have been found than it is not surprisingly to find none of them in the organic phase.

The results of the pre-experiments are complex. But they can not be easily projected onto a disinfection experiment. During the course of disinfection different inherent changements can occur (e.g. chemical reaction, random collisions between particles) coincidental or as a result of the executed steps during a disinfection experiment (e.g. quenching, adding of ClO_2 solution) and thereby trigger the formation of aggregates. To take these circumstances into consideration a standard disinfection experiment was performed and samples were taken at distinct time points. As described in section 3.4 the average particle diameter stayed the same regardless of the moment of sampling.

Before assuming that these results reject Hypothesis 2 it is necessary to consider the inherent properties of them. As described in section 1.7 the resulting diameters are an average of all particles independently of their status (e.g. free, aggregation) and the maximum particle diameter that can be measured with the used apparatus is limited to 10 μm. An explanation of these results could be the non-existence of aggregates in the experimental solution or the presence of only a few aggregates and a lot of free particles.

That is where the necessity of performing additional disinfection experiments (sonication, filtration, ...) arises. Beside such treatments or changes of the experimental solution before starting, also experiments during a disinfection run were perfomed.
All of these treatments should lead to a break up of possible aggregates – already existing as well as evolving in the course of the inactivation process.

Langlet et al. showed that *MS2* aggregates lead to a decline of the counted pfu because each aggregate, independent of its size, cause only a single pfu.
Based on these observations the break up of the aggregates should result in a higher number counted of pfu.

4.3.2 Filtration of the experimental solution

Experiment 2.1 To exclude possible existing aggregates between virus particles that had been observed [37] the experimental solution became sterile filtrated before starting the disinfection process. If these intrinsic aggregates cause the Tailing phenomenon or at least influence it than removing of them should alter the disinfection curve. As described in section 3.5.2 Experiment 2.1 no alterations have been observed.
According to this observation aggregates as reason for Tailing a very unlikely.

4.3.3 Sonication of the experimental solution before starting the experiment

Experiment 2.2 The sonication of the experimental solution before starting the disinfection process was an attempt to destroy possible intrinsic aggregates between virus particles as shown by Hejkal et al.. Also in this case the supposition was that such a pre-treatment should influence the course of the disinfection curve if aggregates cause or at least influence Tailing behaviour. As in section 3.5.3 Experiment 2.2 described no alterations of the disinfection curve have occured. Tailing behaviour could still be observed.
Regarding the results the existence of aggregates can be rejected as explanation for the Tailing phenomenon.

4.3.4 Chloroform extraction

Experiment 2.3 Another attempt to destroy possible aggregates was $CHCl_3$ extraction of the used virus dilution before starting the disinfection. This treatment should ensure that a high amount of virus particles are

present in dispersed form [38].

As described in section 3.5.4 Experiment 2.3 this pre-treatment does not induce an alteration of the course of the resulting disinfection curve. The Tailing behaviour still stayed the same.
Because of the received results the existence of aggregates can be excluded as explanation for the Tailing behaviour.

4.3.5 Higher Sodium chloride concentrated buffer

Experiment 2.4 The aim of this experiment was also to increase the fraction of dispersed virus particles. Therefore three disinfection runs each with a different NaCl concentration were performed. As Sigstam et al. have shown a higher ionic strength leads to a decrease of aggregated particles and consequentially triggers an increase of dispersed particles.

The results are described in the previous section 3.5.5 Experiment 2.4. No changes of the disinfection curve were observed. Hence, no aggregates are present in the experimental solution thus the consequence of this is that Tailing can not stem from aggregates.

4.3.6 Sonication of the experimental solution after 120 s

Experiment 2.5 Once again the experimental solution became sonicated to destroy aggregates but this time during the disinfection process. The choosen time point lay in the region between the end of the transition range and the beginning of the Tailing range. If aggregates are formed during the disinfection process and are not an intrinsic property than this treatment should influence the Tailing behaviour and prompt an alteration of the disinfection curve.

As already described in section 3.5.6 Experiment 2.5 the gained disinfection curve still indicates Tailing behaviour. Due to that results formation of aggregates during the disinfection process can be ruled out as the reason for Tailing.

4.3.7 Lower virus concentration on ice

Experiment 2.6 To decerease the probability of random collision that could trigger aggregation between the virus particles this experiment has been performed with lower virus concentration. Due to the inactivation rate in this special case the technical opportunities are not sufficient to perform an experiment with such low virus concentration. Therefore the whole disinfection process has been performed on ice to slow down the inactivation.

As described in section 3.5.7 Experiment 2.6 two runs with two different virus concentrations were performed. Regardless of the displaced courses of the received disinfection curves, owing to the lowered temperature, the Tailing behaviour remained unchanged. These observations are in accordance with experiments that have already been conducted [22].

The interpretation of the received results are that aggregates are not abundant in the experimental solution and consequently can not explain the Tailing phenomenon.

4.4 Hypothesis 3 — Existance of a resistant virus subpopulation

4.4.1 Washing pause during the experiment

Experiment 3.1 To verify the existence of a more resistant subpopulation the attempt has been to isolate them and observe their resilience.

For the isolation a standard experiment has been used. In the area between the end of the transition range and the beginning of the tailing range the experiment was stopped so that the majority of the sensitive subpopulation can be seen as inactivated. To study their resilience the remaining experimental solution was washed to get rid of the quenching agent and provide a clean experimental solution. Then the disinfection was restarted again.
The course of the restarted disinfection should behave differently from the already performed part before the washing pause because of the huge majority of the more resistant population. As already described in previous section 3.6.1 Experiment 3.1 the resulting disinfection curve was completely contrary to that assymption. In fact the disinfection curve before and after the washing pause shows nearly the same course. In other words the Tailing behaviours still stayed the same regardless which assumed subpopulation has been disinfected.

These observations argue for the absence of a more resistent subpopulation.

These results stand in complete contrast to the observations of Hornstra et al.. The reason therefore in all likelihood can be found in the different experimental designs. They performed a very similar disinfection experiment but with the difference that no washing procedure has been carried out. But this discrepancy could also be an important step in disclosing the phenomenon. Because the conducted washing procedure seems to trigger any alterations that are the reason whether Tailing can be observed or not. What exactly the reasons are can only be guessed but as in the course of the washing the experimental solution becomes changed further investigations should focus especially on it.
The identification of the root cause could be the key solving the Tailing phenomenon.

4.4.2 Changing the concentration of the adding solution

Experiment 3.2 To influence mainly the possibly existing subpopulation the ClO_2 concentration of the added solution (Solution 2) has been changed during this experiment. The time point of change to a higher concentrated solution has been placed into the region between ending of the transition range and starting of the Tailing range. If at least one additional subpopulation with a higher disinfection tolerance exists the higher concentrated solution should lead to a higher disinfection rate in that subpopulation.
This influence should alter the course of the detected disinfection curve.

As already described in the section 3.6.2 Experiment 3.2 before, such alterations have not been observed. Also in the presence of a higher ClO_2 concentrated experimental solution Tailing behaviour is present. A possible explanation could be that the choosen concentration was too low to affect the subpopulation or that no heterogenity in the virus population exists.

5 Conclusion and Outlook

To verify the appearance of Tailing behaviour during the disinfection of bacteriophage *MS2* by ClO_2 disinfection, experiments have been conducted (**Experiment 0.1**, **Experiment 0.2**).

All studied virus stock solutions showed Tailing. To uncover the secret behind this Tailing phenomenon, several experiments were performed. They were used to test three proposed hypotheses.

Hypothesis 1 — Loss of biocidal properties of the experimental solution

Hypothesis 2 — Aggregation of virus particles

Hypothesis 3 — Existance of a resistant virus subpopulation

5.1 Hypothesis 1

Two different experiments were performed to verify the hypothesis. The first was to examine the influence of the ClO_2^- built during a disinfection run (**Experiment 1.2**). The second focused on the virus by inserting additional virus particles in the time range when Tailing started (**Experiment 1.1**).
In both, no changements of the disinfection curve were observed. The Tailing behaviour stayed the same.
$\Longrightarrow$ Hypothesis 1 has to be rejected.

5.2 Hypothesis 2

Disinfection and DLS experiments were carried out.

To gain an insight into a possible formation of aggregates during a disinfection experiment, DLS experiments were conducted. Before the experiments dexcribed above was performed, pre-experiments were used to acquire knowledge about the average particle diameter in the experimental solution at different conditions. In order to perform the disinfection run, samples have been taken at distinct time points. The average size measured of all taken samples has been in the range of the diameter of a single virus particle, which means that no aggregates have been observed.

Regarding the disinfection experiments, different variations of the Standard experiment were performed. All with the aim to break up any aggregates.
For two experiments, sonication was used to destroy possible aggregates. In one case (**Experiment 2.2**), sonication was performed before starting a run. In the other case (**Experiment 2.5**), the experiment was stopped in the time range when Tailing started, sonication was applied, and afterwards the disinfection process was continued..
Another attempt was to filter the experimental solution before starting a disinfection run (**Experiment 2.1**).
Also the salt concentration of the VDB was increased for one experiment (**Experiment 2.4**).
Further, $CHCl_3$ extraction of the added virus stock solution was performed before starting a run (**Experiment 2.3**).
Beside changing of the experimental solution due to alternate buffer composition or different treatments, an experiment that focused on the amount of virus particles was performed (**Experiment 2.6**). Therefore, the virus concentration used was decreased. To ensure useable results, the experiment had to be performed on ice to reduce the disinfection rate.
During all experiments, Tailing behaviour of the disinfection curve has been observed.
$\Longrightarrow$ Hypothesis 2 has to be rejected.

5.3 Hypothesis 3

To investigate the existence of at least two different virus subpopulations, two different experiments were performed. In both, the aim was to deal with the more resistent residues.
To isolate them, a disinfection run was stopped in the beginning of the Tailing range. After washing the remaining solution, the disinfection experiment was restarted (**Experiment 3.1**).
Another strategy was to increase the concentration of the ClO_2 solution that has been added during the experiment. To influence the more resistent subpopulation in particular, the changing of the described adding solution was started in the beginning of the Tailing range (**Experiment 3.2**).

In both experiments Tailing behaviour of the disinfection curve was found.
$\Longrightarrow$ Hypothesis 3 has to be rejected.

5.4 Additional Hypothesis

In the end, none of the three hypotheses could be accepted after considering the received results. This suggests a need to search for other explanations.
Chiefly, Hypothesis 3 seems to be a pivot point for the clarification of the Tailing phenomenon, especially the results of the experiment with a washing pause (**Experiment 3.1**).

Based on the study of Sigstam et al. – in which most of the presented experiments in this thesis contributed to – another hypothesis arises.

"Changes in virus properties: The disinfection process changes virus properties such that they are increasingly protected against the disinfectant" (Sigstam et al., 2014, 85)

Therefore, they performed a standard disinfection experiment with a washing pause like **Experiment 3.1**. But to gain a better understanding about the processes that take place during disinfection on the particle surface, they subjected samples to Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry (MS). More precisely, they studied samples before and after the washing pause to determine any changes in the mass of the viral capsid protein. The results clearly revealed a shift of the capsid protein peak after treatment with ClO_2 compared to untreated control samples. After washing, the capsid protein peak shifted back.
These MALDI MS results indicate that mass changes on the virus particle surface happen during a disinfection experiment have a protective affect. Furthermore, this protection can easily be removed by washing with buffer. The observed masses on the surface have not been identified in detail so far. The authors suggest adsorbed ClO_2^- as the reason for the emerged mass peak, but they point out that further investigation is needed.

The conclusions that arise from these observations lead to the very bizarre circumstances that, in this special case, inactivation leads to the protection against inactivation. This is due to the fact that the disinfection process itself triggers the development of shielding changes on the surface of the viruses.

Finally, these observations uncover the secret behind the Tailing phenomenon – at least for the disinfection of *MS2* by ClO_2 in the applied concentration range. In the field of water disinfection with ClO_2, such knowledge is of crucial importance to provide a proper treatment.

However, further work has to be done. To gain a broader understanding, it would be interesting to conduct the experiments in other concentration ranges, also other viruses should be investigated.
Another important aspect is the identification of the adsorbed substances on the virus surface. Maybe if more knowledge is discovered, Tailing could already be avoided before disinfection treatment is applied.

Appendices

List of Abbreviations

$CHCl_3$ Chloroform

$CaCl_2$ Calcium Chloride

Cl_2 Chlorine

ClO_2^- Chlorite

ClO_2 Chlorine dioxide

ClO_3^- Chlorate

H_2O Water

$K_2S_2O_8$ Potassium persulfate

N_2 Nitrogen

NH_3 Ammonia

$Na_2S_2O_3$ Sodium thiosulfate

$NaClO_2$ Sodium chlorite

NaCl Sodium chloride

NaH_2PO_4 Sodium dihydrogen phosphate

PO_4^{3-} Phosphate

E. coli *Escherichia coli*

MS2 *Enterobacteria phage MS2*

DBP Disinfection by-product

DLS Dynamic Light Scattering

dsRNA double-strand Ribonucleic Acid

LB Lysogeny broth

MALDI Matrix Assisted Laser Desorption Ionization

Milli-Q water Ultrapure water

mRNA messenger Ribonucleic Acid

MS Mass Spectrometry

NA nucleic acid

PEG Polyethylene glycol

pfu plaque forming unit

pI Isoelectric point

RNA Ribonucleic Acid

RT Room temperature

ssRNA single-strand Ribonucleic Acid

THM Trihalomethanes

UV Ultraviolet

VDB Virus dilution buffer

Vis Visible

WHO World Health Organisation