



Swedish University of
Agricultural Sciences

*Inactivation of Viruses in water by chlorination
using bacteriophages as model organisms.*



VIRUS I VATTEN – SKANDINAVISK KUNSKAPSBANK

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Abstract

Climate change will lead to more extreme rain events in parts of Sweden resulting in sewage overflows and thus more contamination of surface water, leading to an increased risk of waterborne infections. Swedish and Norwegian surface waters are rich in humic substances with high organic carbon content that interferes with disinfection, the most important barrier to viruses in the water treatment plant. Therefore the disinfection potential of free chlorine was evaluated for a number of viruses: canine adenovirus (CAV), murine norovirus (MNV), mammalian orthoreovirus (MRV) and porcine enterovirus (PEV), and model viruses (MS2, 28B, Φ X174 and Φ 6 bacteriophages) on different water qualities (pH, turbidity and organic carbon content).

Viruses were enumerated by end-point titration and bacteriophages with plaque assay. The inactivation studies were done by chlorination (The amount of sodium hypochlorite added was 30–60 ml/L based on the types of water) at 5°C and the chlorine values were in between 0.12–0.2 mg/l range. Virus inactivations were determined for the initial reduction (IR) when the chlorine is consumed and further during the contact time within the test tube. During the later part, the chlorine contact time (CT) for one log reduction was determined (CT-1). The initial reduction was in between 3.18–3.94 logs for viruses (Adenovirus > Orthoreovirus > Norovirus > Enterovirus) and for bacteriophages it was in between 0.34–4.89 (Φ X174 > Φ 6 > 28B > MS2). Due to high initial reduction the number of results used for determination of CT for one log reduction were limited. The CT-1 were around 0.5 mg*min/L for all the viruses. For a certain CT-1 time was more important than the chlorine concentration for MNV, PEV and MRV. The CT-1 for the bacteriophages MS2, 28B and Φ X174 were 12.6, 3.44 and 0.79 mg*min/L respectively. The CT values for Φ 6 bacteriophage could not be calculated because of its fast inactivation. MS2 and 28B bacteriophages were persistent to free chlorine and can preferably be used in larger scale pilot studies to better determine the effect of water quality parameters. The Φ X174 had a similar CT-1 as viruses, however the high initial reduction requires that it is added after the initial consumption to be used as a model organism. The fact that time seems to be more important than chlorine

concentration for virus inactivation indicates that the design of contact reactors are important to provide as long minimum contact time for the water to react with free chlorine.

Key Words: Disinfection, Chlorination, Bacteriophages, Viruses, Free chlorine.

1. Introduction

World health organization (WHO) has identified waterborne infections as a major health risk to humans (Fewtrell et al., 2001). According to WHO about 5.7 % of the global diseases are caused due to the poor sanitation and neglected hygiene. Usually the infected individuals will excrete enteric pathogens in large numbers, which causes contamination to others. Diarrheal diseases due to poor water and sanitation leads to an estimated 2.4 million deaths every year (Guerrant et al., 2002). A large part of the population in Sweden and Norway are supplied by drinking water from surface waters (lakes and rivers). These surface waters are often rich in humic substances with high DOC (dissolved organic carbon). In recent years some major outbreaks have occurred due to heavy contamination of the surface water, sometimes in combination with failure in the treatment plant, for example 2004 *Giardia* outbreak in Bergen (Landvik, 2015), 2010 *cryptosporidium* in Östersund (Widerström et al., 2014) and in 2008 a large outbreak of norovirus in Lilla Edet (Larsson et al., 2013).

Climatic conditions will lead to extreme weather events (rain) with sewage overflows and waste water contamination of surface water. The climatic behaviour such as warming temperatures, rise in sea levels and change in frequency of hydrological cycle leads to more floods, heavy contamination is likely to occur more often (Patz et al., 2008). A large number of waterborne outbreaks have been reported from the Nordic countries recently (Guzman-Herrador et al. 2015), summarized in Table 1.

Table 1: Overview of waterborne outbreaks in Nordic countries from 1998-2012 (Guzman-Herrador et al. 2015).

Country	Number of outbreaks	Outbreaks per year	Number of people involved
Sweden	59	4.2	52,258
Norway	53	3.5	10,483

Denmark	4	0.27	660
Finland	59	3.9	22,594

Note: For Sweden, the data is for 1998-2011.

Most outbreaks were caused by caliciviruses however most infection were caused by the chlorine resistant protozoan parasite *Cryptosporidium* (Guzman-Herrador et al., 2015).

1.1. Waterborne viruses

Enteric viruses are most commonly transmitted feacally-orally and through person-to-person contact (Semenza and Menne, 2009). The other common ways of transmission are through drinking, preparation of food or consumption of infected food. Enteric viruses are considered to be emerging water borne pathogens based on their cellular and molecular structures, because of this structure these viruses are resistant to treatment processes. These enteric viruses are usually transmitted by fecal-oral route and replicates in gastrointestinal tract and infect the host organisms. Some of the enteric viruses, which cause water borne diseases are Norovirus, Enterovirus, Reovirus, Adenovirus, Astrovirus, Hepatitis A and Hepatitis E virus (Leclerc et al., 2002) are shown in the Table 2.

Enterovirus:

Enteroviruses belongs to the family *Picornaviridae* contains single stranded RNA with an icosahedral capsid ranging from 20-30 nm in diameter and is responsible for several diseases (Fong and Lipp, 2005). Enteroviruses have a single stranded positive sense RNA and also a protein capsid (Minor, 1996). Many enterovirus infections are asymptomatic that produce mild symptoms such as gastrointestinal infections, abdominal, headache, sore throat and muscle pain. However some enteroviruses may cause more severe infections such as polio, meningitis, meningoencephalitis, acute flaccid paralysis, haemorrhagic conjunctivitis, myocarditis, and pericarditis (Abzug, 2014). Coxsackievirus B5 is a type of Enterovirus that can cause respiratory infections, gastrointestinal illness but also insulin-dependent diabetes and heart diseases (Sinclair et al., 2009). Echoviruses are generally less infectious than other enteroviruses and usually associated with respiratory diseases (Fong and Lipp, 2005). The Enteroviruses of type 68-71 are responsible for the diseases like bronchiolitis (disease of bronchioles), acute hemorrhagic

conjunctivitis, meningitis and paralysis (Abzug, 2014). The common colds are caused by Rhinovirus (30-80%), which infects the respiratory tract. Poliovirus is also a type of enterovirus, which cause poliomyelitis in children. It can also affect the central nervous system leading to paralysis.

Norovirus:

Noroviruses belonging to family *Caliciviridae* have a non enveloped single stranded RNA and has approximately 7.7kb RNA in length and encodes 3 open reading frames (Daughenbaugh et al., 2003). This virus has gained much attention when the impact of gastroenteritis epidemics was seen in Lilla Edet Sweden in 2008 and made 2400 people sick (Larsson et al., 2013). An outbreak of gastroenteritis identified at a hotel in Italy 2005 (Rizzo et al., 2007). Norovirus outbreaks are most common in winter and early spring and it is reported that there are at least 23 million cases of food borne illness in few countries and it is suspected that more than 80% cases are caused by this virus (Hardy, 2005). Norovirus infections impact more than 20 million individuals every year in U.S alone, which result in 56000-70000 people hospitalization and 800 deaths and in developing countries the mortality among children may exceed 200000 per year (Kim et al., 2015). Out of the six genogroups (GI-GVI), GI and GII are most important in causing human infections. Norovirus are primary cause of epidemic and infectious gastroenteritis among all ages of people (Kim et al., 2015). It causes many diseases like vomiting, nausea, abdominal cramps, and non-bloody diarrhea. Many studies on Norovirus indicate that it is resistance to temperature (Seo et al., 2012).

Adenovirus:

Adenovirus belongs to the family *Adenoviridae* and has a non-enveloped double stranded DNA. Adenovirus causes symptomatic diseases of several organ systems such as respiratory diseases (acute respiratory disease), gastrointestinal tract (gastroenteritis), eye (conjunctivitis), central nervous system (meningoencephalitis) and genitalia (urethritis) (Crabtree et al., 1997). The transmission of this virus is through fecal routes and also through human body fluids. Adenovirus has 51 serotypes and all these viruses will cause more or less the same type of symptoms and diseases that will infect adults and children (Schmttz et al., 1983). Among the Adenovirus serotypes, types 40/41 are enteric pathogens that cause 5-20 % of cases of acute gastroenteritis

among infants and young children's. These viruses are sensitive to chemical disinfection but they are more resistant to UV light than other enteric viruses (Rodríguez-Lázaro et al., 2012). A recent Europe-wide surveillance study of these two enteric viruses in recreational waters shows that about 47% of marine and 20 % of fresh water samples contained infectious adenovirus (Wyn-Jones et al., 2011).

Rotavirus:

Rotavirus belonging to the family *Reoviridae* contains 10 segments of double stranded RNA. Rotaviruses have a non-enveloped icosahedral capsid (T-13) with outer and inner protein shell. It is transmitted through fecal-oral route. Rotavirus mainly infects the gastrointestinal tract and is the most important agent of severe diarrhea in children causing more than 500000 deaths yearly in developing countries (Tate et al., 2012). In the year 2008 due to this rotavirus infection 453000 deaths were reported in children younger than 5 years, mainly in countries like Democratic Republic of the Congo, Ethiopia, India, Nigeria, and Pakistan (Tate et al., 2012).

Hepatitis A:

Hepatitis A belongs to the family *Picornaviridae* have a non enveloped single stranded RNA and contains a spherical virion with 27nm, with 7.5kb linear, positive sense RNA (Cristina and Costa-Mattioli, 2007). The genome consists of 5' noncoding region, large open reading frame and a 3' non-coding region. The Hepatitis A virus is responsible for the diseases like Hepatitis, gastroenteritis, abdominal discomfort and jaundice (Konduru et al., 2008). HAV causes approximately about 40% of all acute viral hepatitis. Hepatitis A serotype prevalence in population is under 10% in Nordic countries where endemic is low. A slight raise in hepatitis A infections among individuals was observed with no travel history (Niu et al., 1992). The most common way for transmission of this disease is through fecal-oral route, contaminated food and water and also by person to person contact (Konduru et al., 2008). Hepatitis A can be prevented by Vaccination, sanitation and good health. Before clinical illness develops, Hepatitis A virus will be present in the blood of the infected people.

Hepatitis E:

Hepatitis E belongs to family *hepeviridae* having a positive sense single stranded RNA. It is a small non-enveloped virus with a size of 27-34 nm. Hepatitis E virus has an 7.2kb RNA with three different open reading frames ORF1, ORF2 and ORF3, which plays a role in replication, transcription, capsid protein and viral replication respectively (Norder et al., 2009). Hepatitis E causes acute sporadic, endemic viral hepatitis, anorexia and jaundice (Ahmad et al., 2011). Four distinct genotypes of mammalian HEV have been identified so far. Genotype 1 is identified in humans so far except one pig in Cambodia, genotype 2 is identified in humans in Mexico and Africa, genotype 3 is identified and has been isolated from humans, swine, wild boars and rabbits in endemic and non-endemic countries, genotype 4 has been isolated from humans, pigs and deer in Asia (Widén et al., 2011). The prevalence of HEV in Swedish pig population is not known completely. The zoonotic transmission of genotype 3 infections in pigs when compared to humans is studied by Widén et al (Widén et al., 2011). The virus transmission is mainly by faecal-oral route due to consumption of faecal contaminated water and by ingestion of foodborne products from infected animals mainly pigs. Mortality is mainly in pregnant females (Ahmad et al., 2011). The cases with Hepatitis E genotypes 3 and 4 are mainly found in Europe and the ingestion of this virus is from contaminated water. There has been a study on this virus prevalence in Nordic countries (Denmark and Sweden) in recent years and the researchers have found that the number of patients with hepatitis E viral antibodies in blood is increasing (Norder et al., 2009).

Table 2. Different type of viruses that can be transmitted via water and the diseases they may cause.

Virus	Disease	Reference
Enteric adenovirus 40 and 41	Enteritis	(Rodríguez-Lázaro et al., 2012)
Calicivirus (incl. Noroviruses)	Enteritis	(Larsson et al., 2013)
Coxsackievirus	Various; respiratory illness; enteritis; viral myocarditis; viral meningitis	(Abzug, 2014)
Echovirus	Aseptic meningitis; encephalitis; often asymptomatic	(Abzug, 2014)
Enterovirus types 68-71	Meningitis; encephalitis; paralysis	(Abzug, 2014)
Hepatitis A	Hepatitis - fever, malaise, anorexia, nausea, abdominal discomfort, jaundice	(Konduru et al., 2008)
Hepatitis E	Acute sporadic, fever, epidemic viral hepatitis, jaundice, anorexia	(Ahmad et al., 2011)
Poliovirus	Poliomyelitis – often asymptomatic, fever, nausea, vomiting, headache, paralysis	(Abzug, 2014)
Rotavirus	Enteritis	(Tate et al., 2012)

1.2 Model organisms:

Since not all human viruses can be cultured and cannot be used in pilot and large scale studies. Model organisms or indicators are types of organisms used to detect and quantify the levels of contamination of water. These organisms are not hazardous to the human health but are used as indicators to assess the presence of a health risk or the reduction of a pathogenic virus over a treatment barrier.

Bacteriophages are type of viruses that infects and replicates within bacteria were discovered by Felix D’Herelle and Frederick Twot (Trudil, 2015). Bacteriophages are used as model organisms in the inactivation processes of virus because of their constant presence in sewage and found in

human feces. The structure, size, morphology and the behavior of the bacteriophages in aquatic environment resembles to the enteric viruses (Grabow, 2004). The main groups of the bacteriophages are considered as potential to various aspects regarding water treatment are

- a) Somatic Coliphages
- b) F-specific RNA bacteriophages

Somatic coliphages are heterogeneous belonging to *Myoviridae*, *Podoviridae*, *Macroviridae* and *Siphoviridae* families. F-specific RNA bacteriophages are homogenous belongs to *Leviviridae* family, which are shown in table 2.

The Bacteriophages used in the experiment were MS2, 28B, Φ x174 and Φ 6. MS2 phage belongs to the family *Leviviridae* consists of positive sense and single stranded RNA, which infects *E.coli*. MS2 phage was the first to be sequenced completely. These phages will only infect *E.coli* expressing F pilus (Fiers et al., 1976). 28B bacteriophage belongs to the family *Siphoviridae* consists of double stranded DNA. In terms of morphology 28B belongs to flagella-specific χ species of enteric phages. 28B bacteriophage is recognized to be pathogen for *Salmonella typhimurium* type5. The 28B has not been occur naturally in environment nor in feces but it is used as model organism in several studies (Höglund et al., 2002). Φ x174 belongs to the family *microviridae*, which consists of positive sense and single stranded DNA and it was the first DNA based sequenced genome done by Fredrick Sanger team in 1977 (Sanger et al., 1977). Φ x174 bacteriophage is non-enveloped with simple icosahedral structure (Dika et al., 2015). Φ 6 phage belongs to the family *Cystoviridae* and contains double strand DNA with a tripartite genome with lipid layer in outer coat (Huiskonen et al., 2006).

The animal model organisms used are canine adenovirus, murine norovirus, porcine enterovirus and mammalian orthoreovirus. Canine adenovirus type 1 (CAV-1) belongs to the family *Adenoviridae* contains ds DNA. The CAV-1 virus causes hepatitis, a severe systemic acute liver infection in dogs (Balboni et al., 2015). Porcine enterovirus belongs to the family *Picornaviridae* with single stranded RNA and these viruses are ubiquitous. These viruses occur in swine and the infections are asymptomatic. Based on their cytopathic effect produced in cell cultures, the serotypes of the enterovirus are divided into three groups (I, II and III) (Honda et al., 1990).

Mammalian orthoreovirus belongs to the family *Reoviridae* contains double stranded segmented RNA. The reovirus is responsible for mild illness such as cold and diarrhea mostly in children's. Mammalian orthoreovirus type 3 is used as model organism in the present study, the mammalian orthoreovirus type 3 Dearing strain is currently used as naturally onco-lytic virus used in clinical trails (Kim, 2015). Murine norovirus is used as model organism for human norovirus studies. Murine norovirus belongs to the family *Caliciviridae* family with single stranded RNA. 80 % of the gastroenteritis outbreaks occurred by the norovirus. All these model viruses are used because they share most of the biological properties with the human viruses and are cultured according to the protocol mentioned in methodology section. The different characteristics of the viruses and model organisms used in the experiment are shown in the table 3 below.

Table 3. Characteristics of the different viruses and model viruses used.

Virus	Family	Genome^a (kb)	Size (nm)	Isoelectric point^b (pI)
Human norovirus	<i>Caliciviridae</i>	ssRNA ~7.5	27-32	5,5-6,0
Human Adenovirus	<i>Adenoviridae</i>	dsDNA ~36	70-100	4,5
Human Enterovirus	<i>Picornaviridae</i>	ssRNA ~7.5	27-30	4,0-6,4
Human Rotavirus A	<i>Reoviridae</i>	dsRNA ~18.5	60-70	8,0
Canine Adenovirus	<i>Adenoviridae</i>	dsDNA 30-36	70-90	ND
Murine norovirus	<i>Caliciviridae</i>	ssRNA 7,4-8,3	28-40	ND
Porcine enterovirus	<i>Picornaviridae</i>	ssRNA 7,4	~30	ND
Mammalian orthoreovirus	<i>Reoviridae</i>	dsRNA ~23,5	60-80	ND
Phage 28B	<i>Podoviridae</i>	dsDNA 38-42	60-65	ND
Phage ΦX174	<i>Microviridae</i>	ssDNA, 4-6	~30	6,6
Phage MS2	<i>Leviviridae</i>	ssRNA, 3,6	22-28	3,9
Phage Φ6	<i>Cystoviridae</i>	dsDNA ~13,5	~85	ND

^a Double stranded (ds) or single stranded (ss), length (kilobases/basepairs)

^b When the pH of the virus particle is uncharged, ND = not determined

1.3 Drinking water Treatment

Surface water from lakes, rivers and reservoirs needs to be treated before it has the required water quality. The surface water typically contains highly suspended solid particles and microbial contamination with viruses, bacteria, algae and organic matter. In order to reduce organic and microbial content, the water must be treated through several stages to provide complete safe drinking water. The most commonly used methods by water treatment plants for surface water are filtration, flocculation, sedimentation, and disinfection. Primary treatment is a type of treatment in which suspended solids are removed from the raw water. In Primary treatments of drinking, water viruses are less efficiently removed so disinfection is the critical process for reduction of viruses in surface water. There are several disinfection processes for inactivation of microbes, however Chlorination and UV light disinfection are the ones most commonly used. Based on the considerations of water borne infections, WHO set a target to produce safe drinking water for public health. To produce safe drinking water generally the water treatment plants removes and inactivate viruses to acceptable levels (approx. 10^{-6} per liter). The treatment need for moderately contaminated surface water is normally 6-log reduction, which produces 99.9999% reduction of pathogens in water.

Log reduction: Log reduction is defined as number of microbes removed from the surface by disinfection or by cleaning. For ex: a six-log reduction means lowering the microorganisms by 1,000,000 fold. If the surface having 1,000,000 pathogenic microbes, the reduction of these pathogenic microbes to one gives a six log reduction (Vries and Hamilton, 1999).

The relationship between log reduction and percent reduction is as follows

- 1 log reduction = 90% reduction
- 2 log reduction = 99% reduction
- 3 log reduction = 99.9% reduction
- 4 log reduction = 99.99% reduction
- 5 log reduction = 99.999% reduction
- 6 log reduction = 99.9999% reduction

This means increase in log reduction is more efficient in removing the microorganisms.

Log reduction can be calculated using the formula

$$\begin{aligned}\text{Log reduction} &= \log_{10}(A) - \log_{10}(B) \\ (\text{or}) \\ \text{log reduction} &= \log_{10}(A/B)\end{aligned}$$

Where A = Number of viable microorganisms before treatment,

B = Number of viable microorganisms after treatment.

The treatment of the surface water by the water treatment plants that remove contaminants are as follows,

1.3.1 Conventional Treatment (Flocculation and filtration processes)

Pretreatment of water is carried out to modify the microbial water quality by removing algal cells and protozoan cysts. It involves coagulation, flocculation, sedimentation and rapid filtration. The coagulation removes dirt and particles suspended in water. The floc formed by addition of chemicals to water attracts dirt particles. The floc and dirt becomes heavy to sink at the bottom during sedimentation to clear water flow to filtration. The water passes through filters such as gravel, charcoal and layers of sand helps in removal of smaller particles. These processes results in 1–2 log removals of viruses, bacteria and protozoa. Coagulation conditions such as dose, pH, temperature and turbidity affect the efficiency of removal of microbes. Chemical flocculation and filtration processes will lower the pathogens as well as turbidity and dissolved organic carbon (DOC). The average microbial removal of this treatment process is 27 to 74% for viruses, 32 to 87% for bacteria and 0 to 94% for algae. The efficiency of removal of MS2 bacteriophage and poliovirus are removed at 2.6–3.4 logs. In sedimentation process, coagulated and flocculated particles are removed physically (Fewtrell et al., 2001).

1.3.2 Disinfection

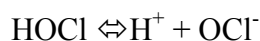
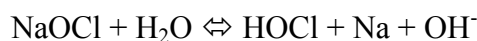
Disinfection is process that inactivates harmful organisms in water. The disinfectants used for chemical disinfection of water are chlorine (Cl_2), chlorine dioxide (ClO_2), hypochlorite (OCl^-), ozone, metals (such as copper and silver), soaps, detergents, several acids and bases, and most commonly used disinfectant is chlorine. For physical disinfection the most commonly used disinfectant is ultraviolet light (UV), gamma rays and heat. Since, only 1-2 log reduction is done in conventional treatment, disinfection is a crucial in order to remove the viruses to provide safe drinking water. These disinfectants also remove organic contaminants for microorganisms and these organic contaminants serve as nutrients or shelters. These disinfectants remain actively in

water even after disinfection because they must have a residual effect. The chlorine gas is hazardous and impractical, so based on this calcium and sodium hypochlorite are widely used disinfectants because they are easy to distribute, cheap and safe use and they are effective against most bacterial and viral pathogens. The factors affecting the disinfection process are pH, temperature, contact time, and concentration of the disinfectant used and physical and chemical parameters of the water.

1.3.3 Chlorination

Chlorination is one of the effective methods for inactivation of viruses. The most used disinfectants are free chlorine and chlorine dioxide. According to Swedish legislation, the amount of free chlorine allowed to add is not more than 1 mg/L. If the demand is high means the concentrations will be low. Free chlorine can be used as primary disinfectant and also can be used as secondary disinfectant in distribution systems in water treatment plants. Monochloramine is also used as a disinfectant but not so effective, it is used to prevent the bacterial regrowth in water pipes.

When sodium hypochlorite reacts with water gives hypochlorous acid and this will give hypochlorite ion.



These reactions are reversible and dependent on pH. If the pH is in between 3.5–5.5 then HOCl is predominant, if pH is in between 5.5–9.5 both HOCl and OCl⁻ will exist in various proportions and if the pH is above 8 OCl⁻ is predominant (LeChevallier et al., 2004).

The knowledge of virus functions is necessary to understand and to predict the inactivation mechanisms, which are unclear. The mode of entry of viruses into the host organism is important, which is different from bacteriophages. Modifications in viral proteins and nucleic acids occur when exposed to inactivating oxidants and radiation. A systematic understanding of virus inactivation losses to functionalities such as host recognition, genome replication would develop understanding and optimization of treatments (Wigginton et al., 2012). Both the HOCl (hypochlorous) and OCl⁻ (hypochlorite ions) are known as free chlorine. HOCl will affect the

many cellular process by producing physiological lesions and also involves in hydrolysis, oxidation and variety of chemical substrates. HOCl upon oxidative decarboxylation converts several α -amino acids into nitriles as major product and aldehydes as minor product. Chlorination of these several compounds gives N-chloro compounds (Pereira et al., 1973). Since the effect of HOCl is more deleterious than ClO^- the oxidation process is pH dependent. Generally the microbes will be inactivated after addition of disinfectants, which means the initial reduction will be seen within very less time. These disinfectants will get into contact with the pathogens at certain time and at a particular concentration. This process of calculating the inactivation rate of the microorganisms is known as CT value.

CT values: The disinfectants will get into contact with the pathogens at certain time with a certain amount of concentration. This process of calculating the inactivation rate of the microorganisms is known as CT value. The CT value is defined as the concentration of disinfectant (free chlorine) and contact time. It is expressed in $\text{mg} \cdot \text{min} / \text{L}$ (Pereira et al., 2013).

$$\text{CT value} = \text{concentration of disinfectant} \cdot \text{contact time} (\text{mg} \cdot \text{min} / \text{L})$$

2. Aim of the study

The specific goals of this project were as follows,

- To study the efficiency of chlorination (free chlorine) on viruses in waters from Scandinavian drinking water treatment plants in laboratory scale.
- To check if bacteriophages are suitable models for viral indicators that can be used in pilot and full-scale treatment plants.
- To identify the effect of water parameters on inactivation studies of chlorination.

3. Materials and Method

3.1 Water samples

We obtained 10 different water samples from Lackarebäck water treatment plant (Gothenburg) and 6 different water samples from Nedre Romerike water treatment plant (Norway). Each of these water samples had different parameters like pH, TOC, color and turbidity, which are shown in below table 4.

Table 4: Characteristics of the waters used.

Waters	Type of water	pH	Turbidity (FNU)	Conductivity (mS/m)	Colour (mg Pt/l)	TOC (mg C/l)
G1	Raw water	7.6	0.48	9.4	23	5.6
G2	Filtered	7.3	0.22	12	4	3.1
G3	Filtered	7.4	0.17	12.5	4	3.2
G4	Raw water	7.6	2.8	9.3	19	6.2
G5	Coagulated	7.1	0.23	11.7	4	3.3
G6	Raw water	7.5	1.1	9.5	22	5.7
G7	Filtered water	7.1	0.18	11.8	4	3
G8	Lake water	7.2	0.72	12.3	46	6.9
G9	Coagulated	7.4	0.33	12.2	5	3.7
G10	Ultra filtered	7.4	0.21	11.8	4	3
N1	Raw water					
N2	Raw water					
N3	Raw water	7.2	0.13	8.2	3	1.5
N4	Raw water	7	<0,1	8.7	<2	1.1
N5	Raw water	7.1	0.17	8.4	2	1.6
N6	Raw water					

Note: All the parameters for Nedre Romerike Vannverk waters (N1-N6) were similar as these waters were taken at the same place (raw water intake) but at different time points.

3.2 Viruses and bacteriophages used in the experiment

Four different animal virus strains and four bacteriophages were used in this study. Canine adenovirus I, strain vacc 98, was cultivated on Madin Darby Canine Kidney (MDCK) cells (ATCC CCL-34). Mammalian orthoreovirus type 3 (ATCC VR-232), strain Abney, was cultivated on Baby Hamster Kidney (BHK-21) cells (ATCC CCL-10). Porcine enterovirus I (ATCC VR-670) was cultivated on SK-6 (Swine Kidney) cells according to protocol reported elsewhere (Kasza et al., 1972). Murine norovirus 1, strain SVA/07, was characterized by molecular methods (Müller et al., 2007) and cultivated on RAW 264.7 cells (ATCC TIB-71).

Bacteriophage 28B was cultivated using the host bacteria *Salmonella typhimurium* phage type 5, the Enterobacteria MS2 phage (ATCC 15597-B1) was cultured on *Salmonella typhimurium* WG49 (ATCC 700730), the bacteriophage Φ X174 phage (ATCC 13706-B1) was cultivated on *Escherichia coli* (ATCC 13706), the bacteriophage Φ 6 (ATCC 21781-B1) was cultured on *Pseudomonas syringae* pv *phaesolicola* HB10Y (ATCC) (Adams, 1959).

3.2.1 Propagation of Viruses

The virus was diluted in the cell culture medium (Department of Microbiology, SVA, Uppsala, Sweden) to obtain appropriate concentration. EMEM (Minimum Essential Medium med Earle's Balanced salt solution) was used for MDCK and BHK-21 cells, and DMEM (Dulbecco's Modified Eagle's Medium) was used for SK-6 and RAW 264.7 cells. Then, the virus was inoculated onto cells in 25 cm² cell culture bottles, and after absorption for about 1 h, cell culture medium was added. 20 ml of cell culture medium was added along with 1-2 % fetal calf serum (Life Technologies, Carlsbad, CA, USA) per each bottle. The virus was incubated at $+37 \pm 2^\circ\text{C}$ with cells to obtain cytopathogenic effect. After 80-100% cytopathogenic effect was evident, the cells were frozen and thawed 2-3 times at centrifugation for $2500\times g$ for 20 min to get rid of the cell debris. The virus supernatant was ultra centrifuged in a L80XP (Beckman Coulter, Fullerton, CA, USA) at $53,900\times g$ for 8h at 4°C . The resulting virus pellet was dissolved in Super- Q water (Dept. of Microbiology, SVA, Uppsala, Sweden), aliquoted and kept at -70°C (Dubovi, 2011).

3.2.2 Propagation of Bacteriophages

The bacterial strain as host for particular bacteriophage was cultivated in the nutrient broth at 37°C for 90 minutes to achieve a concentration of 10^7 cfu/ml. The phages were added in 1:1 volumes to

bacterial solution to obtain the same concentration of phages and bacteria in the solution and cultured for 24 hours at 37°C. On second day, the culture was mixed vigorously and placed at 4°C. The following day, the filtration was performed in order to obtain bacteriophages. To filter out the bacteriophages, the culture medium was centrifuged at 2000×g for 15-20 minutes until clear supernatant was obtained. Now, the supernatant was filtered and concentration of bacteriophages was determined.

3.3 Chlorine inactivation procedure

The chlorine solution used was sodium hypochlorite 3.5% Cl₂ in aqueous solution (VWR, Fontenay-sous-Bois, France). The sodium hypochlorite was pre-diluted to 1:100 (0.035%), and from this 300-600 µl was taken to 10 ml of water depending on water type. Pre-studies were performed to know the appropriate amount of hypochlorite that should be added to the water. All the experiments were performed at 5°C inside a biological safety cabinet. All the glasswares used in the experiment were soaked overnight in diluted hypochlorite solution to avoid contamination. CDF-treatment of glassware was performed as follows, dilute NaOCl to a concentration of >5 mg free chlorine/L in deionized water. Soak all the glassware to be used in the solution overnight or for at least 1 hour before starting the experiment and rinse the glassware to be used 5 times in deionized water, and let it dry. Two glass beakers, one with 150 ml of water (for Chlorine inactivation) and the other with 50 ml of water (positive control) were taken. Test tubes with 9 ml of sodium thiosulphate were prepared.

Chlorine Inactivation: 9 ml of virus and 300 µl of bacteriophages were added to chlorine inactivation beaker. After allowing for 2 minutes, the appropriate amount of hypochlorite solution was added to the chlorine inactivation and chlorine readings were noted immediately and after 2, 4, 10, 30 and 60 minutes. For every reading, two samples were collected. A bag of free chlorine was added to one of the samples, shaken for 20 seconds upside down for allowing the chlorine residuals to react with viruses and bacteriophages and after 1 minute readings were noted using the chlorine kit (HACH, Colorado, U.S). The readings were measured by placing the sample in the kit and readings were noted. A bag of total chlorine was added to the other sample, shaken for 20 sec and after 6 minutes readings were noted again. pH values were measured at 0 and 60 min time

points. 1 ml of sample was added into 9 ml sodium thiosulphate test tubes (1:10) at every measuring sampling point to quench the residual free chlorine or monochloramine.

Positive control Inactivation: 3 ml of virus and 100 μ l of bacteriophages were added to positive control beaker. Hypochlorite was not added in this step. Chlorine readings were observed at 0 and 60 minutes. At every reading two samples were collected. For one sample free chlorine bag was added and shaken for 20 seconds upside down for allowing the chlorine residuals to react with viruses and bacteriophages and after 1 minute reading was taken by using chlorine kit. To the other sample total chlorine bag was added and shaken for 20 sec and after 6 minutes the reading was taken down. 1 ml of sample was added into 9 ml sodium thiosulphate test tubes (1:10) at every measuring sampling point to quench the residual free chlorine.

The glass beakers were immediately placed in 5°C incubator after measuring every sampling point. Now the sodium thiosulphate test tubes containing samples were aliquoted into 1 ml tubes and stored in the - 70°C freezer.

3.4 Enumeration of viruses

Reagents/materials: Cells, Virus sample for titration, Cell culture medium, Fetal bovine serum (FBS), L-glutamine 100mM (Sigma, St Louis, USA), 96-well sterile microtitre plates (Nunc, NY, USA), LAF (Laminar Air Flow) cabin (Heto-Holten A/S, Alleröd, Denmark), Incubator for $+37\pm 1^\circ\text{C}$ 5% CO_2 (Heraeus, Hanau, Germany), humidified atmosphere.

Procedure: The host cells for particular viruses was prepared by adding 100 μ l per well in the 96 well microtitre plates using cell culture medium and FBS solution in a Laminar air flow cabin (LAF). L-glutamine was added to cell culture medium to get final concentration of 1mM. The aliquoted virus samples (obtained after chlorine inactivation procedure) were titrated in cell culture medium to tenfold dilution (100+900 μ l), 50 μ l virus dilutions were inoculated with the above-prepared cells in 8 wells per dilution. For positive control, 50 μ l virus was added in 8 wells as cell control in order to compare with the virus dilutions. The cells were plated and incubated at $+37\pm 1^\circ\text{C}$ in 5% CO_2 for 5-7 days until cpe (cytopathogenic effect) was observed. Now these samples were compared with positive control. The positive wells with cpe effect were counted and

virus titration measurements were determined according to Kärber formula (Kärber, 1931) and expressed as \log_{10} TCID₅₀ value.

3.5 Enumeration of Bacteriophages

Plaque assay was used to determine the inactivation of bacteriophages by measuring the concentration of viruses in the sample. In this method total numbers of plaques (pfu/ml) were measured. The analysis was done by preparing particular host strain to each bacteriophage to determine the plaque count. For plaque analysis, at first 2-3 colonies of the particular host strain were added to the nutrient broth in a beaker and allowed to incubate at 37°C on a shaker for 3-4 hours. Pre heated agar was kept in 55°C incubator to maintain the heat. A 10-fold series dilution was done to the particular samples is shown in figure 1. 2-3 ml of agar, 1 ml of host strain and 1 ml of sample were poured onto agar plate. Agar plates were now kept in the incubator at 37°C for overnight for 15-18 hours. Now the plaque count was performed. After counting these plaques, data analysis such as log reductions and ct values were calculated. The graph was plotted using this data in order to know the inactivation rate.

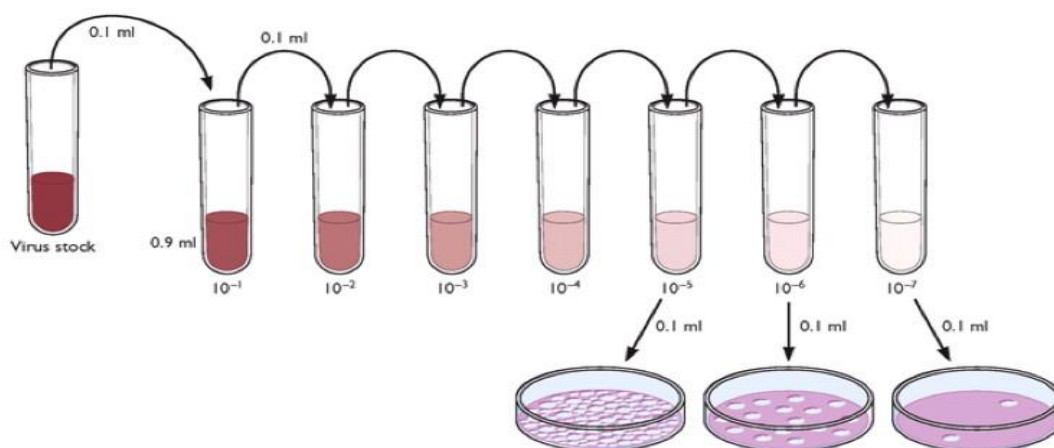


Figure 1: Serial dilutions were performed in Plaque assays (Dulbecco and Vogt, 1953).

3.6 Data Analysis

The CT value is defined as the concentration of disinfectant (free chlorine) and contact time. It is expressed in $\text{mg} \cdot \text{min} / \text{L}$ (Pereira et al., 2013).

$$\text{CT value} = \text{concentration of disinfectant} \cdot \text{contact time (mg} \cdot \text{min} / \text{L)}$$

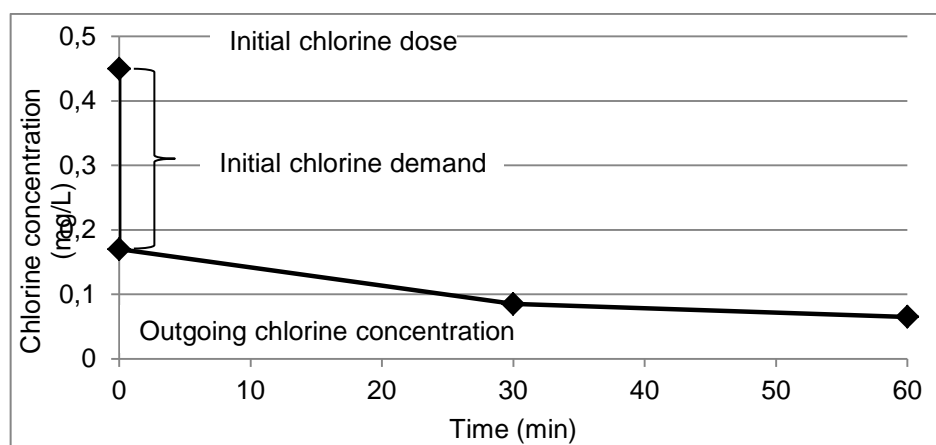


Figure 2: The graph was plotted between chlorine values and contact time for calculating the CT values.

The graph in figure 2 shows the calculation of the CT values by multiplying concentration of the chlorine with the particular time point. These CT values were calculated to obtain certain CT-1 log reduction values. The CT-1 log reduction values were calculated based on the reduction values. The reduction values were calculated by the difference obtained in the log values of plaque forming unit within each sample in order to achieve a certain CT-1 log reduction values. CT-1 log reduction values and initial reduction values were calculated to determine rate of inactivation of microorganism using linear decay kinetics. The initial reduction values were determined by calculating the survival ratio $N-N_0$ of microorganisms, where N stands for infectious viruses at time zero for positive control and N_0 is infectious viruses at time zero for inactivation. Inactivation plots were created to compare CT values, log reduction values and initial reduction values by using Microsoft Excel (Redmond, Washington, Microsoft, 2010, Computer Software).

4. Results

4.1 Inactivation of Viruses

Chlorine inactivation studies were conducted for different types of water samples from Gothenburg and Norway at 5°C temperature and all the water samples had pH around 7. Inactivations were studied at different points in time, which are 0, 2, 4, 8, 10, 15, 30, 45 and 60 minutes. There are no differences observed in the chlorine values between the short time points, so the time points 0, 30 and 60 minutes are considered. The chlorine values of viruses were in between 0.10–0.2 mg/L at 0 minutes and decreases when the time points increase in time and for the positive control the values were 0 mg/L.

The initial reduction values and CT-1 log reduction values are shown in the table 5 below.

Table 5: The average values of Initial reduction values (S.D) and CT-1 log reduction values (S.D) of all viruses. Minimum and Maximum values are also shown in the table.

Virus	Initial log ₁₀ reduction (Avg; std)	Min – max (log ₁₀)	CT-1 log reduction (mg * min/L)	Min – max (mg * min/L)
Adenovirus	3.94 (1.43)	1.87 – 5.68	0.58 (0.31)	0.28 – 0.90
Norovirus	3.48 (0.48)	2.27 – 4.55	0.28 (0.05)	0.22 – 0.34
Enterovirus	3.18 (0.53)	2.20 – 4.30	0.29 (0.17)	0.13 – 0.62
Reovirus	3.48 (0.69)	2.75 – 4.38	0.46 (0.09)	0.39 – 0.61

According to results the initial reduction values are in between 3.18–3.94, which means all the viruses have higher initial reduction values that shows faster inactivation and they died after 2 minutes in most of the water samples and only in few water samples it survived until 10 minutes after addition of free chlorine. The adenoviruses have a greater initial reduction values around 3.94 than other remaining viruses used in the experiment. Adenovirus is least resistant to chlorine among all the viruses as it dies within a few minutes. The initial reductions for some viruses are not calculated because the detection limit values cannot be calculated.

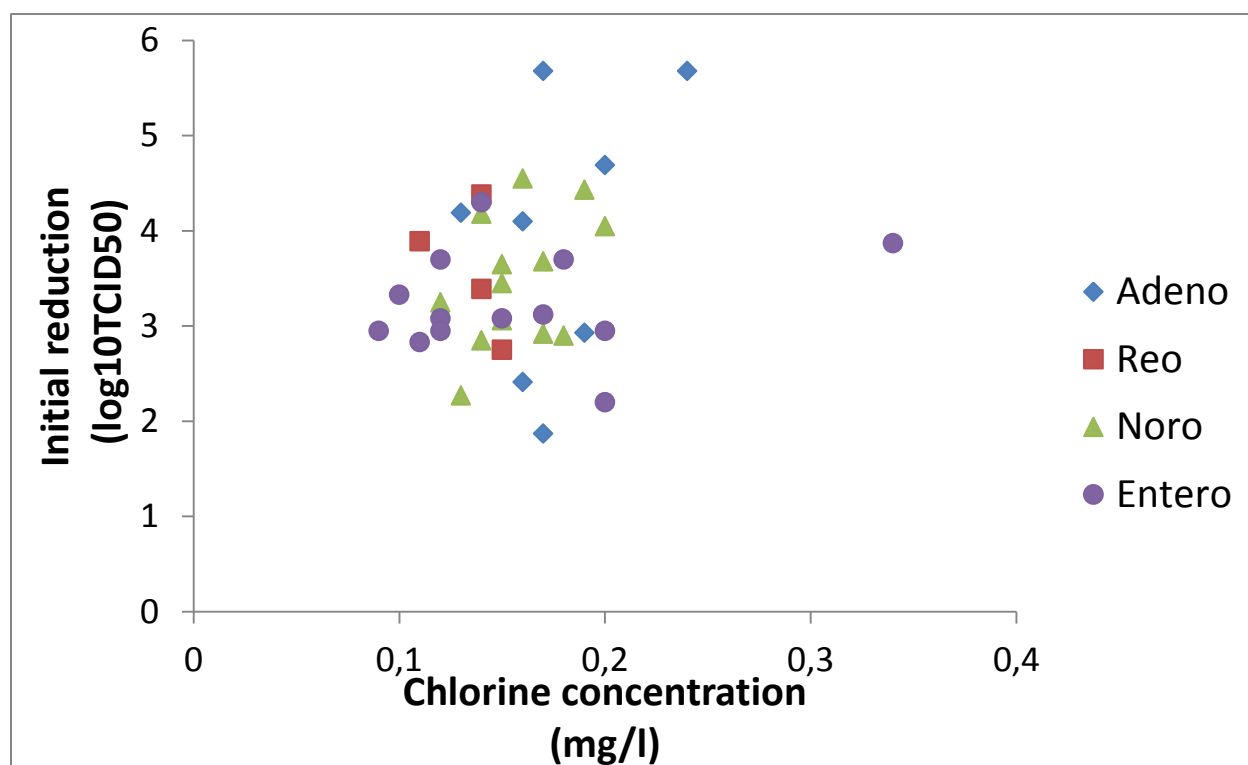


Figure 3: The graph was plotted between initial reduction values and chlorine concentration for the water samples of all the viruses.

The graph in figure 3 shows the initial reduction values versus chlorine concentration. All the viruses used in the present experiment are less resistant to free chlorine, showing higher initial reduction values after addition of free chlorine and the concentration does not affect so much in the end. The Murine norovirus shows increase in initial reduction values with increase in chlorine concentration. All the remaining viruses show almost same range of initial reduction with chlorine concentration.

The adenoviruses have greater initial reduction values in between 4.5–6 among all the viruses at higher chlorine concentration for few water samples with respect to chlorine concentration, which was around 0.2mg/l. Enterovirus and norovirus has an initial reduction value in between 3 and 4 when the chlorine concentration ranges in between 0.12–0.2 for most of the water samples. For some water samples of enterovirus, it has been observed that the initial reduction values were same even around 0.3 mg/l of chlorine concentration. The initial reduction was high, and therefore CT for one log reduction was calculated on a limited number of experiments.

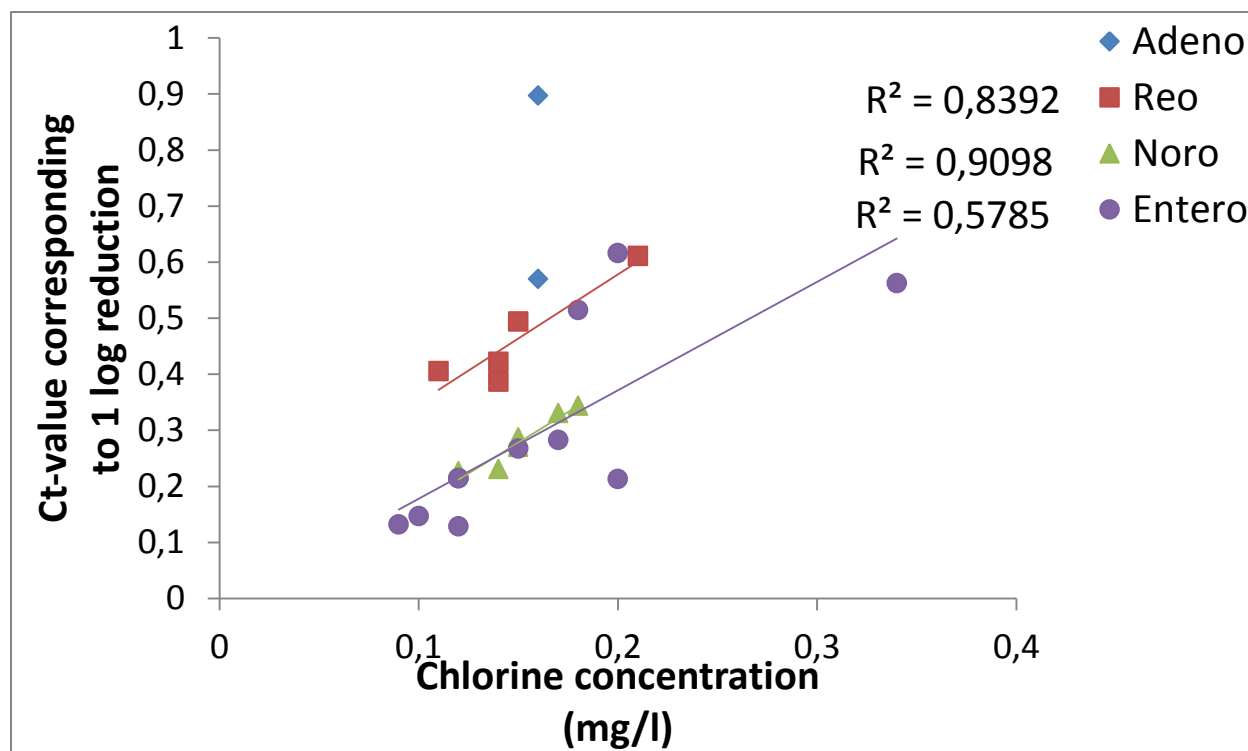


Figure 4: The graph was plotted between CT-1 log reduction values and chlorine concentration of all the viruses.

The CT-1 log reductions were calculated for the viruses, which were shown in the table 5. These values for all the viruses had a mean CT-1 around 0.5 mg * min/L. From the figure 4, the samples with range of chlorine concentration 0.1–0.2 mg/L shows 1-log reduction values. The reduction values were around 1 for most of the viruses and it was not possible to calculate CT-1 log reduction for some water samples. For Enterovirus the values of CT-1 log reduction values are calculated for almost all the water samples expect for a few and the higher range of chlorine concentration also shows the higher CT-1 log reduction values, for enteroviruses. The chlorine concentration is greater than 0.12 mg/L for most of the samples.

4.1.1 Effect of water parameters on virus inactivation

The figure 5 shows the variation of initial reduction values with the pH values. Among all the viruses, adenovirus shows a higher initial reduction values. There were no differences observed in initial reduction regarding with the pH parameter. The pH value of all the water samples were around 7, and the span probably too narrow to be able to measure a difference.

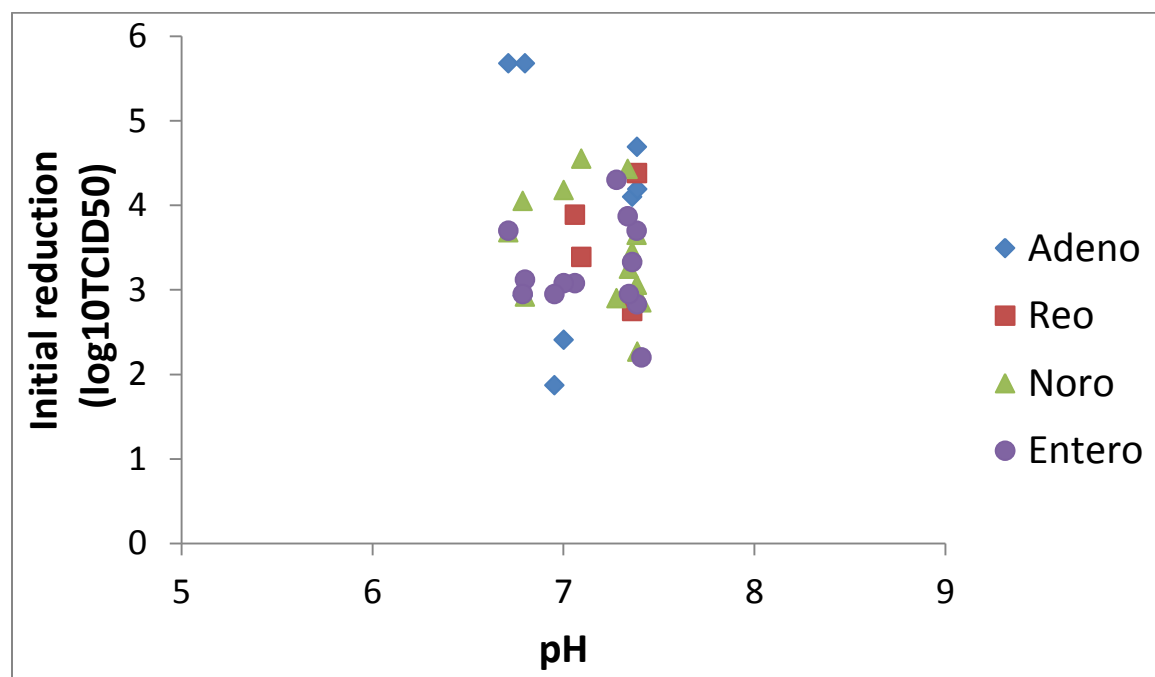


Figure 5: The graph was plotted between initial reduction and pH values between of all the viruses and water samples.

The initial reduction was almost same in all the viruses with regarding the colour parameter. The colour doesn't show any differences between the water samples for the viruses (figure 6). The CT-1 log reduction values were also almost same for all the viruses with this parameter. The sample of adenovirus with higher range of colour shows higher CT-1 log reduction value around 1 and where as in contrast the sample of enterovirus shows low CT-1 log reduction value with almost same range of colour.

Table 6: Initial reduction values (S.D) and CT 1 log reduction values (S.D) of all bacteriophages. Minimum and Maximum values are also shown in the table.

Bacteriophages	Initial log ₁₀ reduction (avg; std)	Min –max (log ₁₀)	CT-1 log reduction (mg * min / L)	Min–max (mg * min / L)
28B	2.14 (0.61)	1.28 – 3.00	3.44 (1.07)	2.41 – 5.12
ΦX174	4.97 (1.97)	2.21 – 6.21	0.79 (0.08)	0.68 – 0.86
MS2	0.34 (0.23)	0.08 – 0.73	12.6 (13.6)	2.87 – 40.4
Φ6	4.89 (0.32)	4.23 – 5.40	n.a	

According to the results obtained MS2 and 28B are more resistant than the other two bacteriophages. MS2 bacteriophage shows low initial reduction values, mean 0.34, and 28 B has 2.14 log₁₀ respectively. The bacteriophages Φ6 and ΦX174 shows high initial reduction values, which are closer to 5. The immediate effect can be seen in the initial reduction for all the bacteriophages. The speed of inactivation was too fast so that CT-1 log reduction values could not be calculated for some samples. The CT values required to achieve 1 log reduction value for MS2 is higher values than all the bacteriophages, which has mean 12.6 and 28 B bacteriophage has 3.4, which is also less persistent to free chlorine than MS2. For few samples of 28 B the reduction value is low, so it was not possible to calculate the CT-1 log reduction values. The CT-1 log reduction of ΦX174 was based on a limited number of experiments and was 0.8. However, this could not be calculated for Φ6 bacteriophage due to its fast initial reduction

4.2.1 Effect of water parameters on bacteriophage inactivation

The water parameters such as colour and TOC play an important role in the inactivation process for bacteriophages. The figure 7 below shows the graph plotted between colour and initial reduction of the bacteriophage 28B. The higher organic load, here measured by colour at 436 nm, had an effect with lower initial reduction.

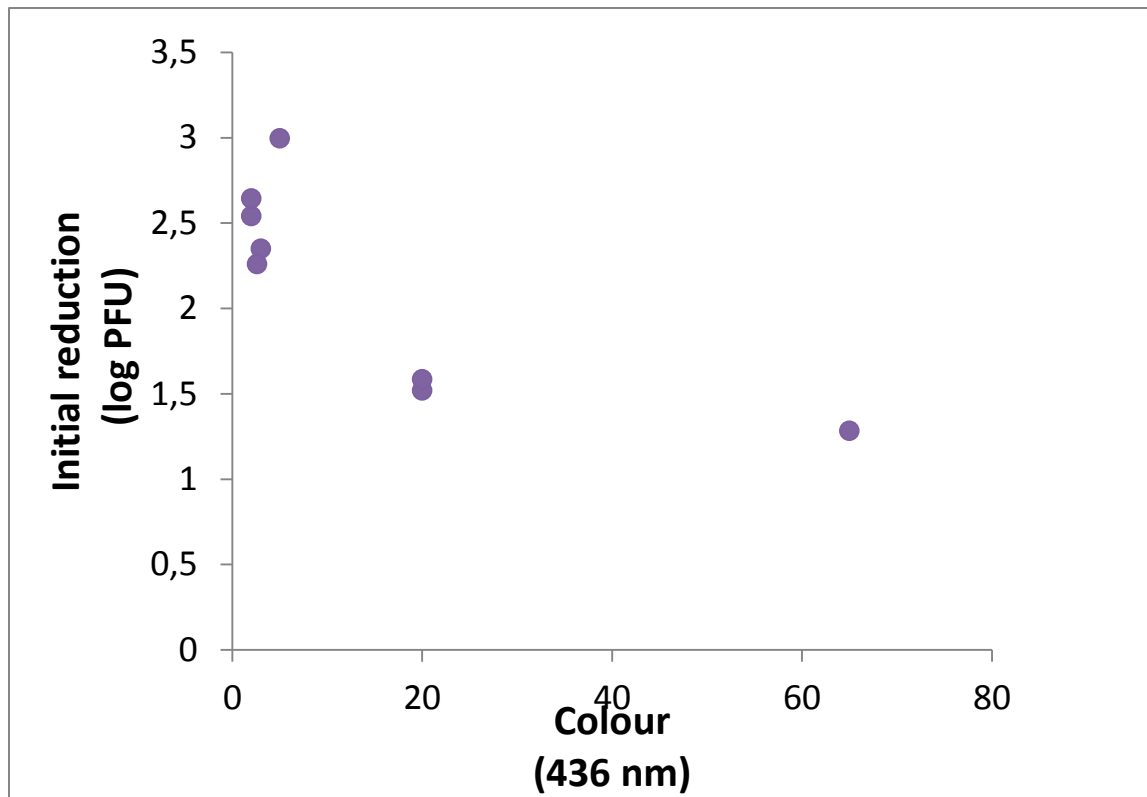


Figure 7: The graph is plotted between Initial reduction values of the bacteriophage 28B and colour.

5. Discussion

In the past decades, enteric viruses, mainly noroviruses, have been associated with large number of water borne infections in Sweden (Guzman-Herrador et al., 2015). Multiple studies have examined chlorine disinfection of enteric viruses. However, these are most often performed in chlorine demand free water, whereas this study was designed to mimic the effect during Swedish conditions.

The results of this research support the idea that the amount of free chlorine added could be higher to react with viruses after the initial consumption. The results presented here are difficult to generalize due to limited number of experiments.

5.1 Efficiency of chlorination on viruses

The results obtained in the present study shows that the viruses are less resistant and inactivated quickly to free chlorine rapidly. The initial reduction, the inactivation taking place when chlorine is consumed by organic material (including microorganisms) was high among all the viruses in between the range 3.18–3.94. Due to the high initial reduction the number of experiments to base the data for CT-1 log reduction was limited. The CT value required to achieve 1 log reduction for adenovirus is 0.58. The study by Thurston-Enriquez et al., 2003 shows that for adenovirus HAdV2 a CT value of 1.5 was required to achieve a 2 log inactivation at pH 8 and 15°C (Thurston-Enriquez et al., 2003) shows consistent with the data obtained in the present study with a CT value of 1.16 (0.58 for 1 log reduction) required to achieve a 2 log inactivation at 5°C. Another study have reported that for HAdV a CT value of 2.6 was required for 4 log inactivation at pH 10 (Page et al., 2009) is also in good agreement with the present study. The CT needed to achieve a 3 log reduction was 3.2 (Kahler et al., 2010) for adenovirus. The time was being an important factor in the present study, the adenovirus died very quickly within 2 minutes for most of the samples and 4 for some samples and it was not possible to calculate CT values for most of the water samples which shows consistency with studies by Kahler (Kahler et al., 2010). For a given CT, time was more important than the concentration (Pettersson and Stenström, 2015). A different study also showed that the inactivation of adenovirus was rapidly done by using free chlorine supports the current study (Baxter C et al., 2007).

The present study determined the CT needed for one log reduction was 0.28 mg*min/L for murine noro viruses. A study by Lim et al., 2010 show that a CT value of 0.31 was required for a 4 log reduction at 5°C and pH 7.2 differs from the present study, which is almost equal to 1 log reduction of our study. The CT needed for 2 log reduction for murine norovirus at pH 7 and 5°C was <0.02 (Cromeans et al., 2010). The chlorine inactivation studies of Murine norovirus could not be evaluated for some of the samples because of its rapid inactivation (Cromeans et al., 2010) agrees with the present study, which dies within less than 10 minutes. The studies of murine norovirus suggest that a CT value of 0.016 (pH 7, 5°C) was required for achieving a 3 log reduction (Kahler et al., 2010). The inactivation of murine norovirus also depends upon the salinity and pH of water, it is more resistant to low pH and inactivated at higher temperatures (Seo et al., 2012). The studies by Cromeans and Kahler show different results with same experimental design for chlorine inactivation studies of adenovirus and murine norovirus (Cromeans et al., 2010).

The CT needed for 1 log reduction of reovirus was 0.46 in the present study. A study also shows that the reovirus was inactivated within very short time (Drulak et al., 1984) agrees with the present, which dies within 4 minutes for most of the samples. A study by Caballero et al., 2004 shows in the presence of 0.2 mg/L free chlorine, no differences were observed in short contact times and whereas at 1 mg/L free chlorine this virus persisted longer in freshwaters. Fewer studies on multiple enteric virus shows that CVB5 was more resistant than the remaining enterovirus types such as E1 and E11. The CT values for these viruses were 0.96 and 0.82 in achieving a 2-log reduction for free chlorine (Cromeans et al., 2010). In the present study the CT for one log reduction was 0.29 mg*min/l of enteroviruses, hence the strain used in the present study was inactivated faster and died within 10 minutes for most of the samples. The enterovirus study showed that the inactivation of enterovirus depends upon the chlorine concentration and a higher chlorine concentration showing faster inactivation (Tree et al., 2003).

The differences in the inactivations could be due to different method and purification of viruses may result in the faster inactivation. The virus used in the experiment can be purified more which can be free from media etc. However, the differences in CT values are due to different experimental designs at various temperatures and type of water quality parameters.

5.2 Bacteriophages as viral indicators

Bacteriophages can be valuable models for studying the inactivation of viruses. Free chlorine studies on the bacteriophages with experimental conditions at 5°C helps us to better understand the bacteriophages as viral indicators. The free chlorine shows less effect on the bacteriophage MS2 when compared to the other bacteriophages. The CT needed for 1 log reduction for bacteriophages MS2 and 28B are 12.6 and 3.44 in the present study, which is a higher CT than for viruses. So, MS2 and 28B are not as suggested good virus models from the data obtained and for the viruses used in the present study (based on the CT values required to achieve 1 log reduction), but MS2 and 28B can be further used to evaluate the effect of different water types in the present study with over a longer time. A study by Tree et al., 1997 also shows that the inactivation of MS2 bacteriophage was poor when compared to other bacteriophages. Some studies also show that MS2 bacteriophage was resistant even at higher chlorine concentrations, but shows an inactivation after 5 minutes and then resistant to chlorine, which is in a good agreement with the present study and the inactivation depends upon dose (Tree et al., 2003) (Sobsey et al., 1989). Another study shows that the CT values required to achieve 2 log inactivation was 0.24 (Lim et al., 2010). Due to the limited number of experiments on these bacteriophages, we could not prove an effect on water parameters except TOC and colour. The chlorine may effect directly on the genome of the bacteriophages, and it also depends upon the structure and size of the genome. The genome size of MS2 bacteriophage is smaller than other bacteriophages, which could explain its better persistence to free chlorine than other bacteriophages.

The CT needed for log reduction of bacteriophage Φ X174 was 0.76, which was closer to the log reduction values of viruses used in the present experiment. The initial reduction is higher for this bacteriophage. As a lot of organic matter was reacting with initial consumption of free chlorine in addition to viruses, this bacteriophage can be used as a model organism if we add it after the initial consumption. The inactivation of Φ X174 was also faster similar to viruses within 3 minutes (Durán et al., 2003). Φ 6 suggested as model for Avian influenza (Adcock 2009), however in the present study the CT values could not be calculated for the bacteriophage Φ 6 as it dies very quickly. The CT-1 log reduction values were not calculated because of the higher inactivation for Φ 6. The findings by Adcock showed that Φ 6 bacteriophage was persistent longer in salinated

water and resistant to chlorine, and can also be used as a model for surrogates in inactivation studies for avian influenza virus (Adcock et al., 2009).

One limitation of using bacteriophages as models for viruses in disinfection processes is that a large part of the effect is on reduced injection of phage RNA into the host cell, which is a different mode of entry compared to that of viruses. However, the fact that there are bacteriophages, which are quite tolerant to chlorine, these can be used to study of the effect of water quality parameters such as TOC, pH and colour. Further bacteriophages can be used for viral inactivation studies in pilot and large-scale studies.

5.3 Efficiency of water parameters on chlorination

The inactivation of viruses and bacteriophages differs between different types of waters under different experimental conditions. In the present study the colour and TOC parameters plays an important role in inactivation studies with viruses, because chlorine was reacting with the water instead of the virus. These water parameters plays an important role for studies on 28B, the lower range of colour shows higher values of initial reduction. A study by Haas et al. suggested that the turbidity might be a factor for lowering the inactivation rate in MS2 bacteriophage in surface waters (Haas et al., 1996). In the present experiment the amount of free chlorine added is consumed by organics present in the water and eventually the concentration will be low. However, a lot of the organic load reacting with chlorine came from the virus stock, therefore a high initial reduction was observed. This high initial reduction is therefore probably not taking place during normal water disinfection. The chlorine consumption during the initial phase gives differences in initial reduction, more used for high coloured water and less for virus stock. But after this initial consumption, no difference was observed apart from time being more important than concentration.

The pH has been shown to have an effect on virus inactivations, being faster at lower pH (Kahler et al., 2010). However this could not be seen in the present study probably because pH range was in between 6.8–7.4, which were too narrow to measure any difference. A study also shows that the inactivation of bacteriophages depends upon the temperature and pH and if the pH is in between the range 6 to 8, the inactivation rate of the bacteriophages are low (Feng et al., 2003). In the present study the pH range is in between 6 to 8, which also aligns with the studies by Feng

with lower inactivation rates for some bacteriophages. Another study also shows that log inactivation shows lower values at higher temperatures (Lim et al., 2010). The type of the water quality from the treatment plant is considered because the stage of treatment of water is important.

6. Conclusions

- **To study the efficiency of chlorination (free chlorine) on viruses in waters from Scandinavian drinking water treatment plants in laboratory scale**

Chlorine efficiently inactivated all viruses in the present study, the CT for 1-log reduction was around 0.5 mg*min/L for all viruses. For a given CT, time was more important than the concentration. Hence design of disinfection contact reactors is important in order to reach as long minimal retention time of the water as possible.

- **To check if bacteriophages are suitable models for viral indicators that can be used in pilot and full-scale treatment plants**

The bacteriophages can be used as model organisms and also to determine the water characteristics in the inactivation studies. Of the tested bacteriophages, ΦX174 can be used as a model organism, which showed the almost same CT value required to achieve 1 log reduction value. However, it should be added after the initial consumption due to high initial reduction. MS2 and 28B bacteriophages were more resistant to chlorine than the viruses, but can preferably be used to determine the effect of water characteristics and in pilot and large-scale studies.

- **To identify the effect of water parameters on inactivation studies of chlorination**

The water quality is an important study for the inactivation studies. In the present study, water parameters do not play any important role except colour and TOC. For the 28B bacteriophage lower range of colour shows higher initial reduction values and higher range of colour shows lower initial reduction values. A large number of experiments for a longer period of time are needed to know the impact of free chlorine on water parameters in detail.

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