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Removal of Salmonella Typhimurium phage 28B by Nanoscale zero-valent iron (nZVI) from water

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ABSTRACT I

ACKNOWLEDGMENT II

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Abstract

Providing drinking water free of viral pathogens is an issue of growing importance in many parts of the world. Historically the focus of water treatment has been on removing bacteria and other pathogens at water treatment sites. The 2 major methods apply for removing parasites and bacteria (filtration and chlorination) do not properly eliminate viruses. Viral outbreaks could be a potential threat for consumers of centralized and decentralized water treatment systems in the case of insufficient treatment. It has been shown in different studies that nanoiron would be efficient to remove a variety of chemical and pathogenic contaminants from water supplies, which opens up the possibility that it could be used for viral removal too. Previous studies have shown that nZVI can work for removal of some of viruses. The object of the present work was to assess the efficiency of commercial nZVI in removal of salmonella typhimurium 28B phage. This virus is chosen as a viral model as it is thermo tolerant and resistant to pH and therefore is a suitable representative for the worst case scenarios.

The experiments were based on column studies where water contaminated with salmonella typhimurium 28B phage was introduced into 3 identical parallel columns with nZVI and three control columns containing only glass beads. Columns were packed in a sandwich form by 2 layers of glass beads which were surrounding nZVI. Slurries were prepared in proportions of 1 to 4 of nZVI powder to water. Applied retention time was 10 min and Q= 2 mL/min. The virus concentrations were estimated by the double layer agar method to count virus mean plaques for influent and effluents water samples. Results indicated a significant (up to 36.85%) reduction of virus concentration in outlets in comparison with outlet. Thus, as based on this pilot study, the use of nanoiron to remove viruses is a technique worthy of further investigation.

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1-Introduction

Providing water free of viruses is a critical issue nowadays. With the growing population and changing lifestyle, there is a higher demand for large amounts of clean water. At the same time, the availability of fresh water is decreasing in some parts of the world as a consequence of global warming. This is likely to result in a scarcity of water in the near future and could be a cause of future conflicts.It has been estimated that by the year 2100 up to one-fifth of the total world population could experience severe water shortages with a 2 °C degree increase of weather temperature (Schewe et al. 2013; Schiermeier 2013).

One possible solution could be reusing treated wastewater, after eliminating pollutants and viruses. However, the current technology for virus elimination in water and wastewater treatment does not come without creating problems of its own. The large amounts of chlorine and ozone required for virus inactivation often result in an unhealthy exposure to disinfection by-products (DBP).For example "Epidemiological studies have suggested a possible link between chlorination and DBP and excess risk of bladder and rectal cancer" (Simpson & Hayes 1998).

In addition, The World Bank has estimated that switching to new water reservoirs as an alternative solution would cost two or three times as much as existing sources since most of the low cost reachable water has been used up (Molle & Berkoff 2009; Serageldin & Mundial 1995).

Last but not least, mutant viruses, like SARC and HIV, are emerging all the time. Viruses, regardless of their genome type that are DNA/ RNA have the highest mutation rate, μ, per generation among all other organisms (Drake et al. 1998), which leads to having a higher possiblity of new viruses among them.So far the main strategy against viral diseases is prevention, due to the absence of a definite chemical treatment for viral disease, comparing to pathogenic bacteria and protozoa.

All these aforementioned issues motivate us to look for new treatments to optimize conventional and sustainable water sources and wastewater treatment that simultaneously meets all of our concerns.

1.1 Pathogenic outbreak

Viruses are an important source of disease, even though it is not known with certainty what percentage is caused by viruses. A paper in 2002 suggested that "Viruses are responsible for 80% of disease outbreaks for which causative agents were identified" (Ryan et al. 2002). However, the published statistics by CDC (Centers for Disease Control and Prevention) for the period of 1991-2000, proposed that viruses accounted for 6% of waterborne drinking outbreaks in US. It is notable that in the presented report, 39 % of the outbreaks agents are undetermined as illustrated in Figure 1(*U.S. Waterborne Disease* 2003).Since viruses are the most challengeable agents for determination among contagious pathogenic agents, it is likely that the real percentage possibly is higher than 6 %.

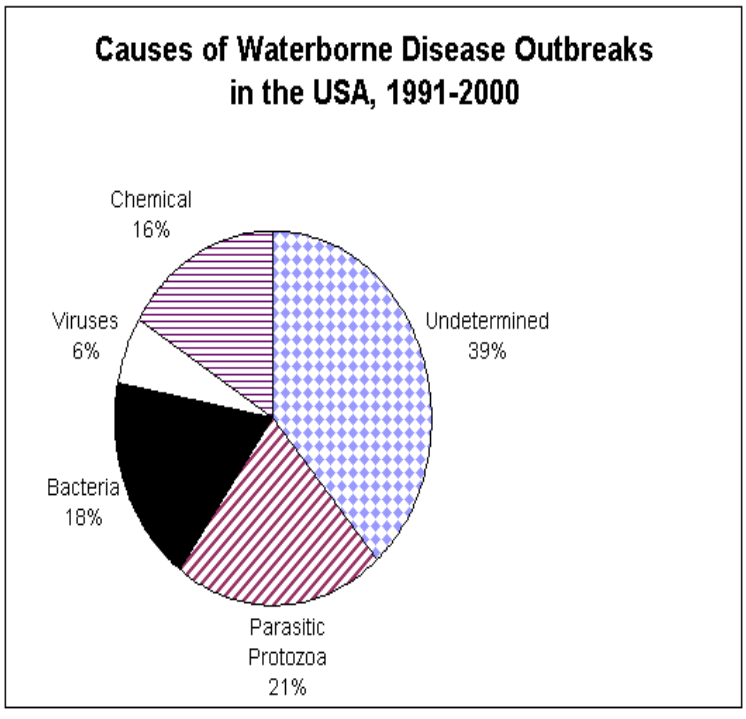

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Figure 1: The causes of 39% of waterborne disease outbreaks are not yet distinguished by current routine facilities. Viruses are recognized as the 3rd pathogenic agent for waterborne outbreaks after Parasitic protozoa and Bacteria among waterborne diseases.

Regardless of the type of the drinking water source, centralized or decentralized, both are susceptible to carry viruses. Groundwater and surface water both would contain viruses and bacteria and subsequently would create infections for consumers; however, groundwater is less susceptible to contain parasitic protozoa than the surface water.

Furthermore, these days the pattern of drinking water supplies is changing from a decentralized to a centralized one. In the new format, the health of a larger population is impacted at same time, which calls for further investigations for virus removal from water supplies.

On the top of that, the type of diseases caused through water is important. Among the viral waterborne pathogens which are transmitted through drinking water, Hepatitis E virus (HEV), Hepatitis A virus (HAV), Adenoviruses, and Enteroviruses are known to lead to severe diseases. Figure 2 illustratestheaforementionedinformation in moredetails.

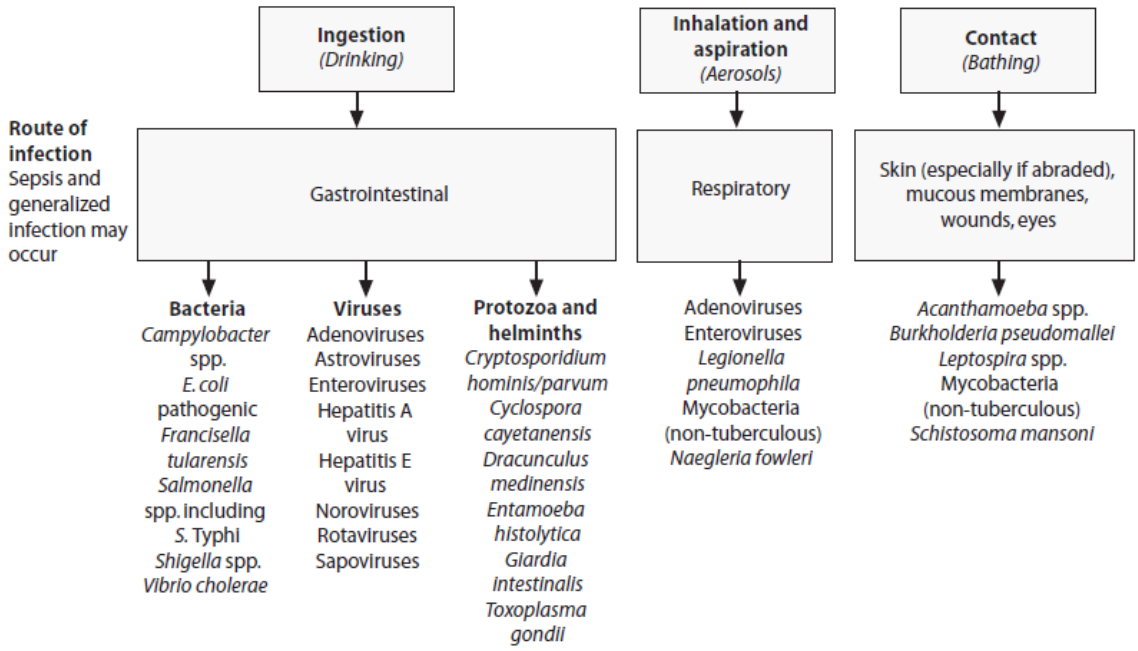


Figure2: Transmission route of dominant waterborne pathogens according to(WHO 2004).

1.2 Importance of virus removal:

As shown in the table1, viruses have high survival rate in water supplies, moderate resistance to chlorine, and high relative infectivity (WHO 2004). Also based on US Ground water guideline, 2007, pathogenic viruses are objective for water quality as well as other waterborne pathogens. It is therefore important to have a sufficient log reduction through an appropriate treatment. For example based on British Columbia regulation, it is recommended to have a minimum of 4-log reduction or inactivation of enteric viruses for surface water supplies.

Table 1. Pathogens transmitted through drinking-water (WHO 2004)

Pathogen	Health signiÞcance	Persistence in water supplies	Resistance to chlorine	Relative infectivity	Important animal source
Bacteria					
Burkholderiapseudomallei	High	May multiply	Low	Low	No
Campylobacterjejuni, C. coli	High	Moderate	Low	Moderate	Yes
Escherichiacoli– Pathogenic[f]	High	Moderate	Low	Low	Yes
E. coli– Enterohaemorrhagic	High	Moderate	Low	High	Yes
Francisellatularensis	High	Long	Moderate	High	Yes
Legionella spp.	High	May multiply	Low	Moderate	No
Leptospira	High	Long	Low	High	Yes
Mycobacteria (non-tuberculous)	Low	May multiply	High	Low	No
Salmonella Typhi	High	Moderate	Low	Low	No
Othersalmonellae	High	May multiply	Low	Low	Yes
*Shigella*spp.	High	Short	Low	High	No
Vibriocholerae	High	Short to long	Low	Low	No
Viruses					
Adenoviruses	Moderate	Long	Moderate	High	No
Astroviruses	Moderate	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Hepatitis A virus	High	Long	Moderate	High	No
Hepatitis E virus	High	Long	Moderate	High	Potentially
Noroviruses	High	Long	Moderate	High	Potentially
Rotaviruses	High	Long	Moderate	High	No
Sapoviruses	High	Long	Moderate	High	Potentially
Protozoa					
*Acanthamoeba*spp.	High	May multiply	High	High	No
Cryptosporidiumhominis/ parvum	High	Long	High	High	Yes
Cyclosporacayetanensis	High	Long	High	High	No
Entamoebahistolytica	High	Moderate	High	High	No
Giardiaintestinalis	High	Moderate	High	High	Yes
Naegleriafowleri	High	May multiply	Low	Moderate	No

The recommended log reduction is calculated to meet an acceptable exposure to enteric virus. This is based on the existence of the minimum virus concentration in surface water, 1 (no. / 100 L) (The Ministry of Health & BC's health authorities 2012). Table 2 illustrates information in details.

Table 2: Necessary virus log removal based on concentration on surface water (The Ministry of Health & BC's health authorities 2012)

Source water virus concentration (no./100 L)	Overall required treatment reduction for viruses (log_{10})
1	4
10	5
100	6
1000	7

Respecting rotavirus (as an indicator) concentration 10 (n/L) at raw water, 5.96 log reduction has been intended to meet a tolerable risk. (Table 3) "A 5.96 log_{10} unit reduction for rotaviruses corresponds to 99.999 89% reduction" (WHO 2004).

Table 3. Example incidence of selected viral indicators and pathogens in faeces, wastewater and raw water

Microbe	Number per gram offaeces	Number per liter in untreated wastewater	Number per liter in raw water
Enteroviruses	10^6	1−1000	0.01–10
Rotaviruses	10^9	50–5000	0.01–100

Consequences of the viral disease depend on the economic level of the target population. Low income areas are more severely impacted. Therefore, WHO suggests a higher log reduction for rotavirus in those territories (WHO 2004).There is thus an important demand for an efficient treatment that does not necessitate the implanting of costly infrastructure in developing areas.

It follows that a number of methods are available for the treatment and purification of water supplies and wastewater, one of which is iron-based remediation technologies. These include nanoiron, which is the topic of the present investigation. But the next section gives an overview of different iron-based purification technologies.

1.3. Iron based purification technologies

Iron is the fourth most abundant element in the Earth's crust (5.63%)(Bibby et al. 2001; Guerinot 2000).Iron has an important role in the geochemical processes at environment and has been recognized and used widely for removal and absorption of impurities or contaminations from water and wastewater. The significant role of iron is noticeable in a number of environmentally based systems of purification. Also, it has been represented by several researchers the inactivation effect of iron species on a wide range of microorganisms. (Kim et al. 2011; Shi et al. 2012; You et al. 2005)

1.3.1 Eco-sanitation (sustainable sanitation)

1.3.1.1Soil filtration

One of the applications of iron in the environment is soil filtration for gray water treatment. In soil filtration, the existence of Fe and Ca and Al are important for absorbing Phosphorus from wastewater(Green & Ho 2005). The removal mechanisms can be summarized as follows: Chemical fixation occurs by soluble Fe at pH<5.5 and fixation by hydrous oxides of Fe, Al, and Mg at 5.5< pH<8. Fe $(PO_4)_2$, $AlPO_4$, Ca_3 $(PO_4)_2$ are products of this reaction and which mostly are found in the oxidized layer, B horizon, of the soil profile. To design soil filtration, the existence of oxidized layer and the indicated elements which make the dark brownish layer, should be considered. The soil profile oxidized layer is illustrated in Figures 3a and 3b.

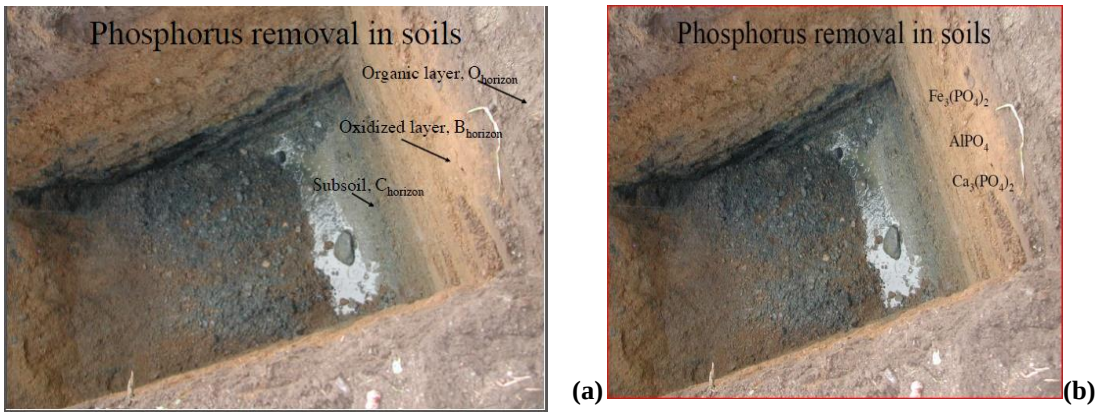


Figure3a, b. Location of the oxidized layer in soil profile (picture has been kindly provided by Professor Peter Jenssenat university NMBU)

Iron plays a role in other sustainable sanitation systems, such as soil mound systems, Constructed Wetland (CW)(Cundy et al. 2008), and to some extent contamination removal can also be explained by the correlation and existence of iron.

Soil mound systems are a type of low-maintenance system which can be enhanced with a variety of materials to facilitate removal of phosphorous(Green & Ho 2005).

The aforementioned system can facilitate ion exchange and also precipitation of phosphorus through provision of increased surface area, chemicals including iron and calcium compounds, and pH(Charles et al. 2008).

Constructed wetland (CW) is a treatment system which cleans and purifies the loaded wastewater passively. The purification takes place on the surface or subsurface of the wetland through the reductive biochemical reactions which iron participates in (Brix 1994; Cundy et al. 2008). It has been also been suggested that the efficiency of wetland treatment should be evaluated with the help of ratios of individual iron oxidation states(Diáková et al. 2006).

1.3.1.1 Filter media

Depending on the physical and geological of the situation, iron could be a natural part of the system or could be added to prepared design specifications. Soil filtration is an example of a natural system.

Largely in advanced grey water treatment system and constructed wetland, iron would be utilized as a part of the added filter media by either choosing sand or Leca with high content of iron. Leca, as illustrated in Figure 4, is an example of a filter media. Leca is a product name of the Light Weight clay Aggregate (LWA), which is clay produced to be dried, expanded and formed into small balls under industrial conditions. LWA is also a popular filter media used in advanced treatment systems in Norway. The surface area of Leca media is > 500m3/m3(Jenssen et al. 2001; Jenssen et al. 1994).

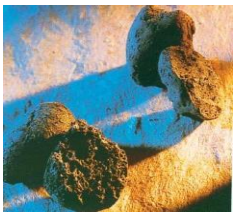


Figure4. Filter media, Leca particles(weber SAINT-GOBAIN 2014)

According to producers the high surface area and high iron content in form of $Fe_2O_3$are specific advantages of Leca (Technical data, 2014).

1.3.2. Conventional treatment

One of the most efficient methods known in conventional treatment is chemical coagulation (Medema et al 1998). Different forms of iron salts have been used widely in water and wastewater treatment plants. Besides aluminum salts, iron salts (Trivalent iron salt, bivalent iron salts) are a common coagulant in the chemical step of water treatment. One of the focusing purposes for that is phosphorus reduction. In addition, good treatment efficiency is guaranteed with iron salts with reasonable price. Iron salts are also beneficial for controlling the odor in wastewater plant sites. Furthermore, the utilization of iron chloride with lime as a sludge conditioning agent is common (Gillberg et al. 2003).

By application of chemical coagulation, we expect a 1-2 log reduction for pathogens (Bacteria, viruses, and protozoa). It has been proved that iron-based coagulants are more efficient to a certain extent than aluminum-based coagulants in removing bacteria and protozoa. (Au &

LeChevallier 2004).But this is not extensible to viruses. In a project by Bell et al (2000) 4 model viruses were treated with 3 coagulants, Ferric, Alum & PACI. In that experiment they found that MS-2 and human enteric poliovirus had a reasonably higher log reduction than phage PRD-1 and enteric echovirus.

Similar observations were made in the other common methods of conventional treatment. For example the efficiency of lime softening on the following viruses, MS-2, Poliovirus and HAV was tested in a project. For bacteriophage MS-2, 2 hours was enough to reach a 4- log reduction with lime treatment; but a similar reduction was reached with HAV by increasing the exposure time to 6 hours. With poliovirus, a 2.5 log reduction was observed after 6h, showing it to be the one most resistant to alkaline condition. Similar conclusions have been suggested for application of slow sand filters (Au & LeChevallier 2004; Ellis & Wood 1985), and granulated gels (Mouillot & Netter 1977) for virus removal.

1.3.3. Iron- based remediation mechanisms

Iron has been employed in remediation field for a long time. The underlying mechanisms can be categorized in two main groups.

Sorptive/ stabilization mechanism

In sorptive/stabilization technology, iron works mostly as sorbent, co-precipitation, and immobilization agent. The technology based on this mechanism is suitable for lands which are highly contaminated with previous industrial wastes or for soil leaching. To change the condition in these situations, iron rich soil would be added to contaminated sites. In such cases,
Fe is considered to work as “an assisted natural remediation”(Cundy et al. 2008). The aim of this iron-based remediation is to decrease the toxicity of the land through inhibition of soil leaching. It is possible that changes in chemical conditions of the site would lead to re-release contaminants into the site in the future. Good arsenic fixation by this method for contaminated areas has been demonstrated(Cundy et al. 2008).

Reductive mechanism

Transfer of an electron from iron to the contaminants can lead to transformation, immobilization, or decomposition of the contaminant to less toxic Species. As an example, Fenton treatment technology is based on the electron donation by Fe^{0} or Fe^{+2} in the presence of H_2O_2via the strong oxidizing agent hydroxyl radical.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^{-}$$

•OH has been introduced as a highly reactive agent for oxidization of organic compound Cundy et al. 2008) and inactivation of wide range of microorganisms (Kim et al. 2010; Kim et al. 2011; Nieto-Juarez et al. 2010; Ryan et al. 2002; You et al. 2005)

Fenton treatment largely is applied for detoxification of pesticides (chlorinated contaminants), fuels and industrial waste. Ground water remediation from chlorinated contaminants is an excellent application of the process.

Low pH requirement can limit the application. However Kakarlaet al. (2002) developed a method with applying chelated–iron catalysts and stabilized hydrogen peroxide to avoid the low pH for in-situ remediation of chlorinated contaminants in groundwater(Cundy et al. 2008).

1.3.4. Nanoremediation

Finally, iron-based remediation can be based on application of nanomaterials. The principle is similar to the sorption and reduction processes described above, but rather than use of bulk or micrometer sized particles, the efficiency of remediation processes can be increased by application of the iron in the form of nano-sized particles. The main process and treatments are described below. But first a general introduction to nanomaterials and nanoparticles.

1.3.4.1 Nanotechnology

Nanomaterials are defined as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm"(European Commission 2005).

Natural nanomaterials are almost found everywhere in nature. They are found in form of organic materials (carbohydrate, proteins, and humic materials), iron oxides and aluminium oxides in the solid part of the soil. Natural nanomaterials are constantly formed and transformed by geological and biological processes.

Incidental nanomaterials are unintended results of human activities, such as smoke or car exhaust emissions(Coutris 2012).

Lastly, *engineered nanomaterial*, are designed nanomaterials which their characteristic makes them to be distinctive from non-nano scaled chemicals of the same composition. On important aspect of their feature is that the reactivity of nanomaterials increases along with the decrease in size of nanoparticle. Going into further details, reveals that as the size of a nanoparticle decreases, more atoms are situated at the surface of the particle. This leads to a higher energy at surface and makes the particle a highly reactive nanomaterial. This aspect has been recognized and applied by researchers in many different fields ranging from energy and drug industry to water purification and remediation (Coutris 2012; Kim et al. 2011; Müller & Nowack 2010; Shi et al. 2012). This is illustrated by the fact that production of nano-materials is rising. Till recently more than 1300 products has been registered by the Woodrow Wilson Nanotechnology Consumer Products Inventory (WWNCPI 2012) cited by (EL-Temsah 2012).

With respect to remediation, engineered nanoamterials has facilitated cost effective remediation technologies compared with other cleanup methods, either because they can be applied in areas which otherwise would be almost impossible, not simply reachable, or too expensive. One of the most widely recognized particles in nano remediation is nano zero-valent iron (nZVI)(Karn et al. 2009).

1.3.4.1.1 Nano Zero- Valent Iron, nZVI

nZVI is an engineered nano scaled zero-valent iron which has a small particle size comparing to other ZVI species (reactive media). These particles are extremely reactive due to their nano scaled size. Among different nano materials, nZVI has been a popular application for research because of their unique characteristics. nZVI could provide a cost effective and fast remediation technology. Injection of nZVI directly into contaminated sites, instead of digging or pumping for ground water remediation by ZVI could make it a convenient application. Availability is another advantage for this product, as it is relatively easily manufactured.

Production of nZVI:

In general nZVI could be produced by two main ways, bottom–up or top-down methods. In the bottom–up method the particles are produced from solutions of atoms or molecules making nZVI through chemical and physical procedures. In contrast, in the top-down method, bulk material is broken down through physical and chemical actions to reach into nanoscale size particles(EL-Temsah 2012).The nanoparticles used in this project came from "NANOIRON", which is a Czech Republic company, producing nZVI via thermal reduction of bullet sized magnetite (Fe_3O_4)(NANO IRON 2010).

Mobility

The mobility of the nZVI is a key feature of its remediation properties. For in situ remediation the particles must be mobile and stable enough to reach the sites before degradation. The applicability of the particles in porous media has been questioned because of very low mobility (Schrick et al. 2004) cited by(EL-Temsah 2012). It has been observed in several column experiments that particles without coating aggregate easily in saturated soil(EL-Temsah 2012).

In a solid phase, nZVI has an affinity to aggregate due to their magnetic properties and extremely small size. The use of transport or coating the particles is a solution to increase mobility in porous media for nZVI particles(NANO IRON 2010). Application of different surfactants as dispersion agents can ensure that nZVI retains its colloidal characteristics. For water remediation, the low mobility can be an advantage in filtration applications, provided that the nZVI retains its chemical properties in filtration media.

2. Study objectives and design

Previous studies have shown that nanoiron is an efficient treatment to remove a variety of chemical and pathogenic contaminants from water supplies, and a few studies have indicated that it could be used for removal of viruses as well (Kim et al. 2011; Shi et al. 2012).On this basis, the main hypothesis tested in the present study is that nZVI can be used to remove *Salmonella typhimurium* phage 28B (S.t.28B) from drinking water. Full details of methods and procedures are given in the following section. But first a few words on the selection ofS.t.28Bas a test virus for the study.

2.1. Bacteriophage

Bacteriophages are like viruses attack and infect bacteria. Based on host cell, three main classes have been defined within virus group which is the plant viruses, animal viruses and finally bacteriophages.

Bacteriophages were discovered during World War I, by two scientists independently: Frederick W Twort in England in 1915 and Felix d'Herelle at the Pasteur Institute in Paris in 1917, and were defined as a new class in virus classification (Pelczar 1988) cited by (Grabow 2004).Very soon attention had been derived to apply phages as a tool in water quality assessment and molecular genetics. This is due to the unique characteristic of phages. While they carry small genome like other viruses; they have simple, feasible and reasonably- priced cultivation and detection methods compared to other viruses.

The amount of bacteriophage measured in various environments are as follows: 10^6 -10^8 per liter in sewage (Bell 1976; Ignazzitto et al.1980; Havelaar & Hogeboom 1984; Havelaar et al. 1984; Tartera et al. 1989 reviewed by Grabow et al. 1993), 10^5 per liter for lake and river water (Goyal et al. 1987), 67 phages per liter in a sewage-polluted river (Tartera et al. 1989).

Different environmental factors have been tested on survival of the phages and virus such as pH, nitrate concentration, temperature, turbidity, or hardness of water. Among those, effect of temperature on virus inactivation and survival has been recognized to be efficient for many years (Niemi 1976) (Yates et al. 1985).

2.2. Model virus selection

The process of selecting a virus is a challenging issue for any study. The selected virus should meet certain criteria proportionate to the aim of the study. Generally, some viruses are more persistent against elimination / inactivation process, thus a selected model virus should be a good representative for those. In other words it should pose "greater challenge to the virus elimination process" (Barbara et al. 2008). Although *"Salmonella typhimurium* phage 28B (S.t.28B) (Lilleengen, 1984) has neither been shown to occur naturally in environmental samples nor in feces"(Höglund et al. 2002)it has been applied in several studies as a model or a tracer microorganism. These include studies on for groundwater flow modeling (Carlander et al. 2000; Johansson et al. 1998), virus transport and mechanisms in porous media (Heistad 2008). It has also been has been employed to trace the source of fecal contamination (Stenström1996), and has been recommended as a model for the risk assessment of human enteric virus (Havelaar1991).

The bacteriophage *Salmonella typhimurium* phage 28B (S.t.28B) is classified as a member of *Siphoviridae* family of which they are dDNA. The host cells of the entire family cells are limited only to bacteria. The genetic sequence of the virus has not been characterized yet (Heistad, 2008)."S.t.28B has an isometric head of 64 nm in diameter and moderately long, thick, tapering tails of 225 nm × 10 nm with 57 or 58 cross-striations"(Eisenstark et al. 2009). In terms of morphological characteristic S.t.28B belong to the flagella-specific χ species of enteric phages though it was not seen to be adsorbed to bacterial flagella. Specifically S.t.28B is recognized to be pathogen for *Salmonella typhimurium*type5.

The S.t.28B virus, also defined as a bio-colloid, is thermotolerant(Eller 1995; Norin et al. 1996) has low inactivation rates at high pH and is considered relatively resistant to alkaline pH (Carlander and Westrell1999). This means that S.t.28B is one of the most conservative microorganisms Lasobrasetet al. (1999) believes that other phages are fragile comparing to S.t.28B.Of course availability was another selecting factor for this phage. Appropriate propagation and uncomplicated detection methods (plaque assay) for S.t.28B are valuable assets. These are described in detail in the materials and methods section.

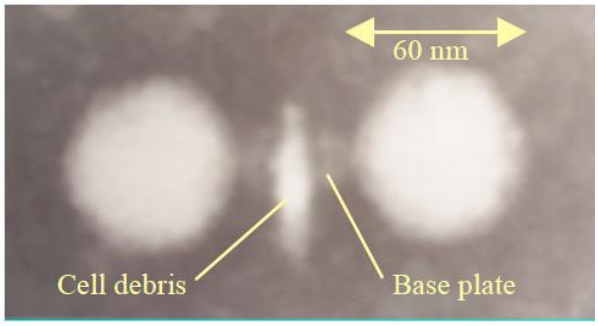


Figure5. Scanning Electron micrograph of Salmonella typhimurium phage 28 B (photo provided by AH, adapted from: K.O. Hedlund, SMI)

3. Materials and method

3.1 -nZVI, NANOFER STAR production from NANO IRON, S.r.o Company at Check Republic

NANOFER STAR (powder) is made from pyrophoric zero-valent iron nanopowder which is a commercial chemical produced by NANO IRON, S.r.o Company at Check Republic. The aforementioned product has an average particle size of 50 nm, with an average specific area of 20-25 m^2/g, a narrow particle size distribution of 20-100 nm and a high content of iron in the range of 80-90 wt %.

To prevent rapid oxidation or combustion of Fe (0) nanoparticles, the particles are stabilized by a thin layer of iron oxide. Thus stabilized Fe (0) particles must be activated in an aqueous environment which required the preparation of a slurry/suspension from nanopowder according to the instructions provided by the supplier. The method of homogenization has an extreme influence on the reactivity of the production in water. High–shear mixing ensures that the particles do not undergo sedimentation to less reactivate micro size particles. Since Fe (0) particles are very reactive/reductive products they will be reacting with oxygen in water and turning into iron oxides and hydroxides over time. This process is called "aging". The most suitable and stable environment for Fe (0) nanoparticles is anoxic and reducing conditions. In this experiment the main challenge regarding nZVI is handling during application

Preparation and handling nZVI slurry

Nanopowder and water were mixed with a ratio of 1 to 4 by high-shear mixer by an ultrasound shaker. This slurry of ¼ proportions was used as the initial stock solution for further dilution.

3.2 The Virus (*Salmonella typhimurium phage 28B*) and the Bacteria (*Salmonella typhimurium type5*):

The S.t.28B and proper host bacteria, *Salmonella typhimurium*type5, samples were provided by Gorel Allestam at the Swedish Institute for Infection Disease Control and subculture was produced and kept in standard conditions at IKBM at Norwegian University of Life Science, NMBU.

3.2.1. Propagation

These series of laboratory operations have to be performed over 3 continuous days.

Day 1: Preparing Media and host culture

Nutrient Broth, (NB) and host culture

Nutrient broth was prepared by dissolving 8 grams of nutrient broth powder (Merk Brand) in 1000 mL MQ water. Then 20 X 5 mL of prepared NB was transferred to 5 X 100 mL E- Flasks and then autoclaved. 1 loop of *Salmonella typhimurium*type5 was transferred into the 100 mL E-Flask and incubated for 4 hours shaking overnight at 37 °C. It is vital to fill part of the cultivation dishes in the indicated format to provide sufficient space for air exchange while they are reproducing.

Nutrient Agar (NA)

The nutrient agar media was prepared by dissolving 23 grams nutrient powder (Merk Brand) in 1000 mL MQ water by heating up to boiling point. The prepared media was autoclaved at 121 °C and was kept in room temperature for further applications.

Nutrient Agar (NA) plates: Approximately 15 mL of melted NA was poured into each petri dish. Plates were dried with the lids off in Microbiological Safety Cabinets under standard conditions.

Soft agar preparation: 20 mL of melted NA was diluted with 20 mL prepared NB. Prepared soft agar was incubated at 45 °C to avoid solidification.

Dilution buffer: Phosphate buffered saline, PBS, was taken off from shelf.

Measuring the virus stock concentration

The concentration of phages in the stock solution has been determined by plaque assay and then restored for further application for the second day at 4-8 °C.

3.2.2Plaque assay, PA, double agar

PA has been applied to determine the concentration of the stock solutions and collected samples from inlet and outlets from all 3 collectors.

A series of 10-fold dilutions of the phage solution has been prepared through a cascade diluting. First, 1mL of phage suspension was diluted with 9 mL of PBS buffer at a tube which was labeled 10^{-1}. For preparing 2nd dilution, 1 mL of dilution 1 (10^{-1}) was transferred to the 2nd tube (10^{-2}) and mixed properly with 9 mL PBS. This process was continued as illustrated in the Figure7 to prepare the entire whole 10^{-1}-10^{-10} dilutions.

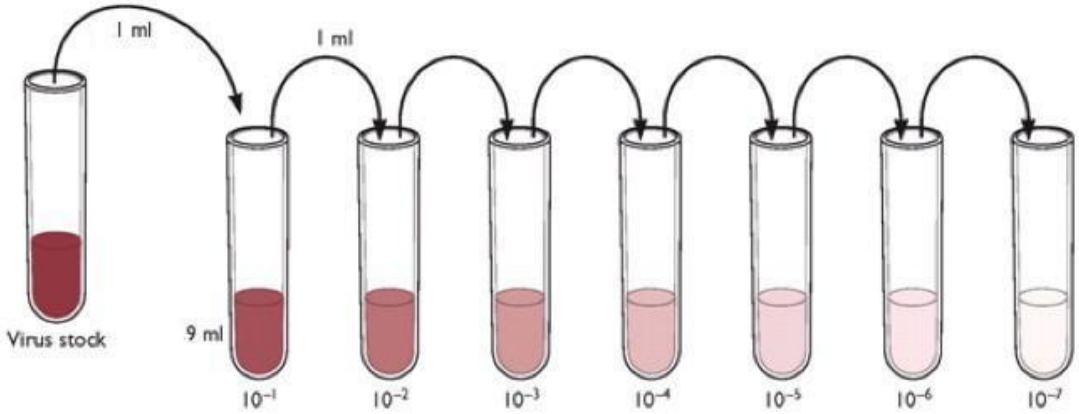


Figure7. Performance of dilution series from virus stock

In next step of plaque assay performance, a 5 mL mixture was prepared. The mixture was contained 0.5 mL phage solution from dilution $10^{-1}$and 0.5 mL cultivated host bacteria in NB and 4 mL SA. The mixtures were spread evenly on the NA plates and were solidified in room temperature. Two plates were prepared from each 10^{-1} -10^{-10}dilutions as explained in previous step (Total 20). Later, all plates were transferred into the incubator and were kept overnight at 37 C overnight (18 hours).

On the day after, all appeared plaque were counted manually. Obtained figures were representing plague forming unite, PFU. For each dilution, 2 PFU numbers were obtained. PFU numbers are in range of 10 <PFU < 100 give the highest degree of certainty. (Dulbecco & Vogt 1953).

Determined concentration was found 2 x 10^9PFU/mL

Day 2: Virus propagation

To make a 10 fold dilution of the overnight incubated salmonella, 2.5 ml of salmonella culture from the first day, was transferred into another 20 ml NB and incubated at 37°C while shaking for 2 hours. After that the bacteria are in the exponential phase. 25 mL of host culture was mixed with NB to increase the volume to 500 mL.

To propagate the virus, we need to add a specified amount of phage to the solution, as calculated based on Multiplicity of infection (MOI). According to MOI for this case, a ratio of 1 to 4 between target bacteria and the S.t.28Bwas required.

Calculation:

Host culture in exponential growth phase contains=	2 x 10^8 cells/mL
Total amount of host in 25 mL=	25 x 2x 10^8
Ratio phage/host =	1/200

Calculated phage concentration on our primary stock solution= 2 x 10^9PFU/mL

Formula

$$\frac{1 \times 25 \times 2 \times 10^8}{200 \times (2 \times 10^9)} = 1.25 \times 10^{-2}$$

1.25×10^{-2} Calculated, then 1250 µL from phage dilution 2 (because of 10^{-2}) were collected and mixed with 25mL host cells and incubated at 37°Cwhile shaking for 10-12 minutes. Then the volume of solution was taken to 500 mL by adding NB and incubation continued for 4-5 hours.

At final step, 5 mL (10 ml per L) Chloroform was added to kill the host cells and release the phage particles. Then the solution was stirred for 10 minutes and kept overnight at 4-8 °C.

Day 3: Final preparation of Virus solution

The whole 500 mL of solution from day 2 was centrifuged at 3000 rpm and the supernatant which containing the virus solution was transferred to a sterilized container. Finally the solution was filtered through 0.45 µm sterile filters to give a clear and pure solution.

The propagation was followed by a plaque assay to determine the virus concentration, after propagation, and during the experiment. Two virus stock solutions were prepared by this method kept in the cold room until beginning of the column tests.

The estimated virus concentrations as determined by the plaque assay method immediately after propagation were as follows:

Stock 1:1.70E+10PFU/mL

Stock 2:2.24E+09PFU/mL

Estimated virus concentration after the storage period (immediately before running the columns):

Stock 1:1.76E+09PFU/mL

Stock 2:2.45E+08PFU/mL

Stock 1 used for preparing the water/phage mixture for column running for both control and nZVI tests at two different time points. This was chosen as our reference stock for making the water/phage mixture for column running. The preparation was done at two different time points for control test and nZVI test. Details are as follows: 1 liter of water/ phage mixture prepared by this equation $C1.V1 = C2.V2$ Where,

V1 = volume of initial solution,

C1 = concentration of initial solution

V2 = final volume of the new solution,

C2 = final concentration of the new solution

The solution was shortly utilized for the column running and in that point final concentration of mixture determined and represented as an inlet concentration. The concentration of both mixtures will be illustrated in the results section.

3.3. Water chemistry

The water used for preparation of slurries and column running was collected from Milli-Q water system. The ionic strength was fixed at 10mM and pH was adjusted to 7.75-7.77.The water was sterile and degassed to prevent additional effect of oxygen on nZVI and inactivation of viruses. It was desirable to have low pH because induced activities by nZVI would lead to produce hydroxide during the experiment which can increase the pH around 4 units. Additionally we must avoid having pH around 8-8.2 as this pH zeta potential would be equal to 0 which for zeta potential will lead to agglomeration of nZVI (the solution with zeta> 30 mV, Zeta <30 mV are stable).

3.4 Setup

Column packing

2 series of columns with 3 repetitions were packed in same way: 3 controls plus 3 nZVI columns.
Control Columns: 3 parallel and independent cylinders designed and packed identically with glass beads to operate under a continuous saturated flow. Experiment was operated with glass beads as the filter media which were well- sorted with average particle size diameter of 0.4 mm. Glass beads were acid washed and autoclaved without background contamination.
nZVI columns: continuous saturated glass beads column were mixed with nZVI(reactive media)in a sandwich form with 3parallel and independent cylinders designed and packed identically also. The columns are transparent glasses cylinders with an inner diameter of 20 mm and length of 200 mm (≈ 18 cm actual length for filling), and were filled under following condition: Sandwich form, two 6.75cm layers of cleaned glass beads on top and bottom surrounding one 4.5 cm layer of glass beads mixed with nZVI. In the middle layer, the glass beads were mixed with nZVI in a volume of 1/1(V/V) which resulted in 15% of total volume for the nZVI finally.
The column setup for running the nZVI test is illustrated in Figure 7.

3.5. Column Running

The figures and calculation for running the solution through the column were as follows:
Retention time: 10 min → Flow Q=1.879 ≈ 2 (mL/min) → 1PV= 20 mL
The proposed set up with the peristaltic pump with continuous up-flow is illustrated in figure 1.First, 10 pore volumes of the background solution (Milli-Q water; with ionic strength= 10mM

and pH = 7.75-7.77) which is free of phages was introduced to stabilize the system and reach to steady state condition (10 PV → water volume = 200 mL, t=100 min). In the next step, at time zero the virus solution with known concentration was pumped into the system for 10 pore volumes and then switched to the background water and continued for another 5 PV(water volume= 100 mL, t=50 min) to make sure that all free viruses were washed out of the system.

The water was collected at time zero from each column separately and analyzed by plaque assay to determine the virus concentration shortly after the collection was completed.

Temperature was kept constant at around 4-8 °C for both container and collectors solution to minimize the effect of inactivation by high temperature.

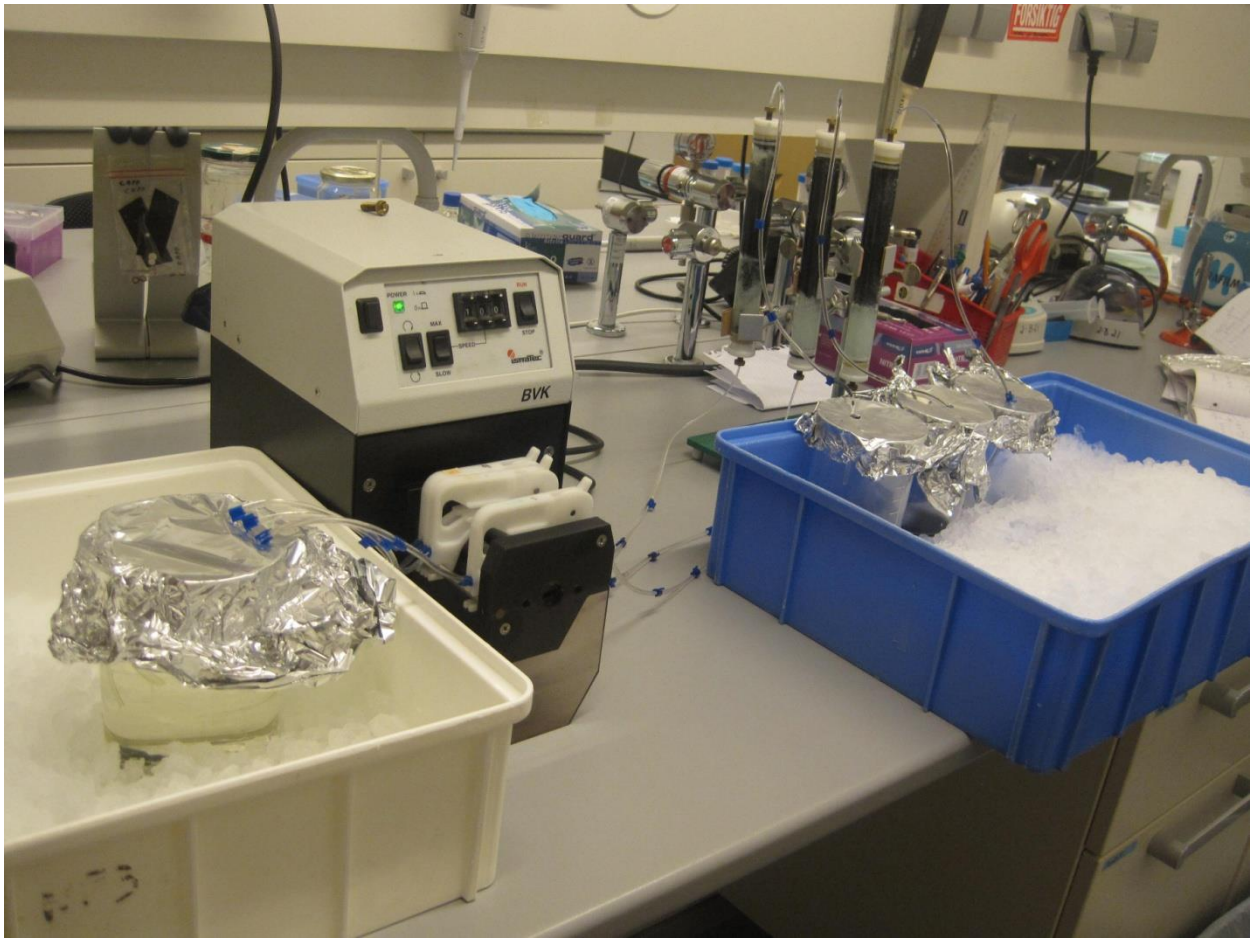
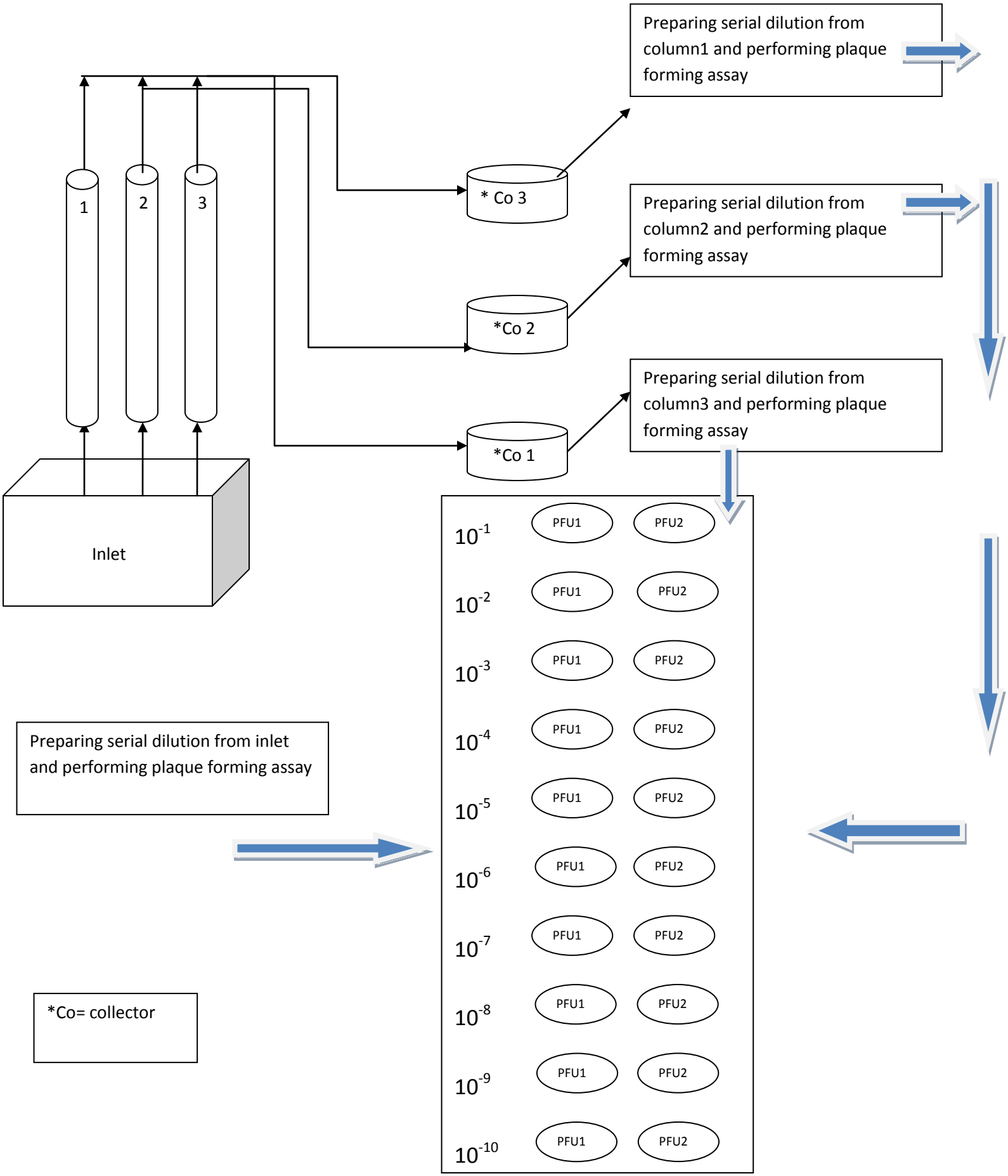


Figure7. Experiment setup for nZVI test. Influent solution, water10mM mixed with S.t.28B, (left side) pumped upward by peristaltic pump into nZVI/glass beads columns. At final point water fraction collectors (right side) are collecting the treated water for 10 PVs separately.

3.6. Statistical analysis and data handling

Statistical analysis was performed using Graph pad version 5. Data was presented as mean±sdbased on three independent replicates. Difference in significance between the treatments was analyzed by student T-test using $p \leq 0.05$ as criteria for significance. Statistical analysis was performed on normally distributed data, while the data not meeting the criteria of normality was $\log_{10}$ transformed prior to analysis.

Flow chart


Preparing serial dilution from column1 and performing plaque forming assay
Preparing serial dilution from column2 and performing plaque forming assay
Preparing serial dilution from column3 and performing plaque forming assay
1
2
3
* Co 3
*Co 2
*Co 1
Inlet
10^{-1} PFU1 PFU2
10^{-2} PFU1 PFU2
10^{-3} PFU1 PFU2
10^{-4} PFU1 PFU2
10^{-5} PFU1 PFU2
10^{-6} PFU1 PFU2
10^{-7} PFU1 PFU2
10^{-8} PFU1 PFU2
10^{-9} PFU1 PFU2
10^{-10} PFU1 PFU2
Preparing serial dilution from inlet and performing plaque forming assay
*Co= collector

4. RESULTS

4.1. Control test

Results from the control test showed that there was no viral removal in presence of glass beads under saturation conditions. The concentrations (as derived from the Plaque Forming Unit (PFU) method, were the same in the inlet and outlet, over a range of different dilutions (Figure 7a). The results of manual PFU counting are shown in Figure 7a. The bars represent the average of the three columns, where two aliquots (PFU1&2) were counted for each column. There was no significant difference between inlet and outlet over a range of 4 dilutions (numbers 5, 6, 7, and 8). The plates from dilution numbers 1-4, were not countable due to high number of emerged plagues. No plaques were detectable in dilution 9 and 10. In dilution number 6 the initial S.t.28B titer(inlet) which introduced into 3 parallel columns is 80.5 +/-7.07E-01 (PFU/mL average) and after passing through glass beads columns has been reached to 80.33 +/-2.73E+00 (PFU/mL average from 3 columns). Because PFU numbers are in range of 10 <PFU < 100 give the highest degree of certainty (Dulbecco, R. and M. Vogt 1953); Dilution 6 in the graph is the most representative which shows no removal through control columns.

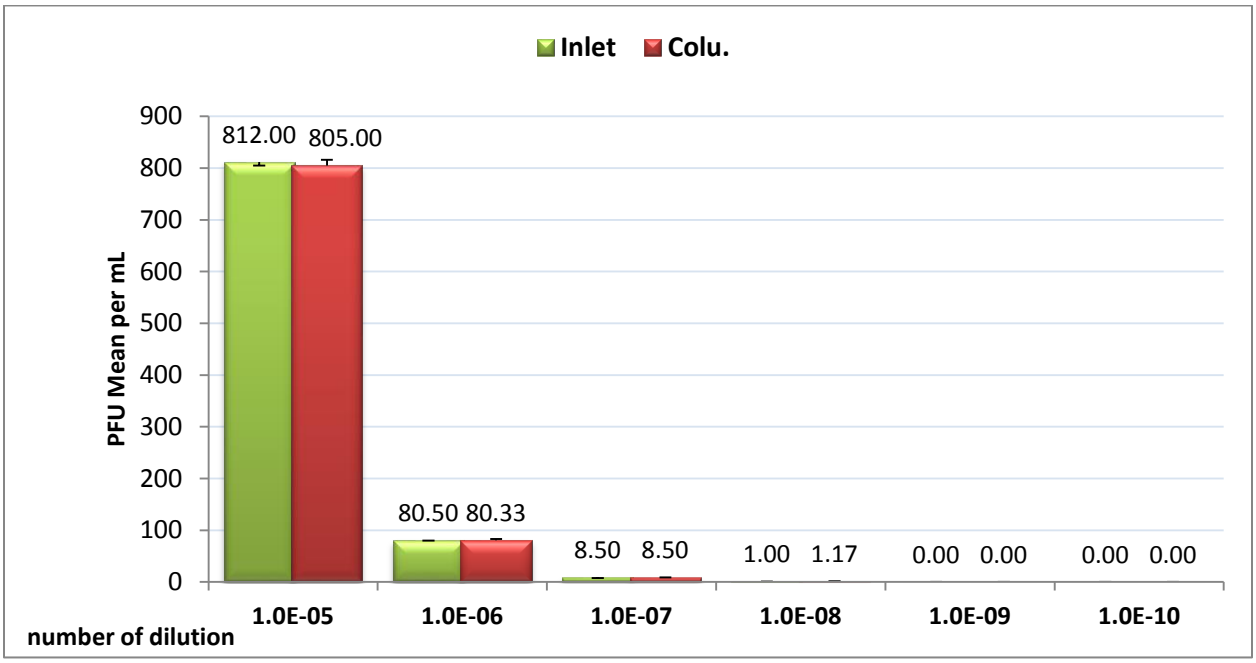


Figure8 a. *Salmonella typhimurium* phage 28B (S.t.28B) concentration as PFU/mL following passage through control columns loaded with glass beads and (pH_0=7.5, Retention time: 10 min, Temperature=4-8 ºC, the medium was10 mM in Mili-Q water for both cases.). n= 3 (3 column replicates x 2 PFU measurements).

4.2. nZVI treatment

In comparison to the control result, the columns loaded with nZVI showed a marked reduction in viral concentration following passage through the column (Figure 7b). A significant reduction in PFU was observed over a range of 3 dilutions (5, 6, 7 & 8).The displayed PFU in the graphs represents the average of the three columns, where two aliquots (PFU1&2) were counted for each columns. As discussed above, from a virologist's perspective, the dilution nr 6 is the most representative as the PFU lies between 10 and 100. At this dilution the virus titer dropped from 85.5 +/-7.07E-01 PFU/mL (average of pfu1& pfu2) at the inlet to 54 +/- 3.90E+00 PFU/mL (average of PFU1&2 from 3 columns) at outlets. Based on that, 36.84% viruses removed/ inactivated which is equal to 0.20 log reduction. No plaques appeared in dilution 9 and 10. From 10 prepared dilutions, the first 4 of them was swamped by plagues and omitted from counting.

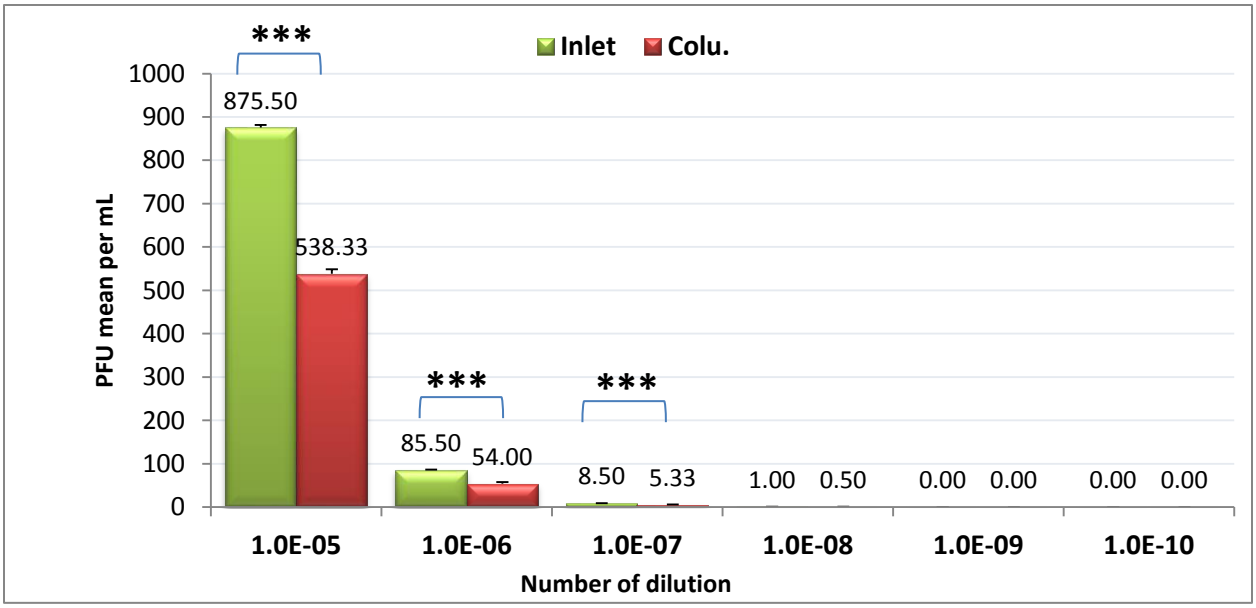


Figure8b.*Salmonella typhimurium* phage 28B (S.t.28B) concentrations as PFU/mL following passage through nZVI columns loaded with nZVI/ glass beads and (pH_0=7.5, Retention time: 10 min, Temperature=4-8 ºC, the medium was10 mM in Mili-Q water for both cases.). n= 3 (3 column replicates x 2 PFU measurements).

4.3. Percentage reduction in virus concentrations

The PFU data can be used to calculate the reduction in virus concentration. The formula employed to calculate virus concentration was presented in the methods section. Detailed information on the numbers can be found in the excel sheets in the appendix file. PFU numbers of dilution 6 has been applied for calculation of virus concentrations to plot the figure 9 and that is why to follow within the expected range of PFU. The plot of log viral concentrations at **Figure 9** also shows no significant difference between virus concentrations for the control test (p=0.3). As shows for control test, the virus concentration in the inlet was 1.61 E+08 (PFU/mL) and 1.61 E+08 +/- 3.06E+06 (PFU/mL) after passing through the glass bead columns (average for 3 columns). The results indicate that glass beads alone are not effective for virus removal from the solution. Also as illustrated in the Figure 9; the average concentration of S.t.28B has been reduced by about 36.84% from 1.71E+08 (PFU/mL) to 1.08E+08 +/- 3.61E+06 (PFU/mL) after passing through nZVI laded columns (15% total volume). Statistically the difference between the viral concentration of influent and nZVI treated virus solution was significant (p>0.01) or (p=0.0001). The results show that nZVI is effective for removal of S.t.28B from solution.

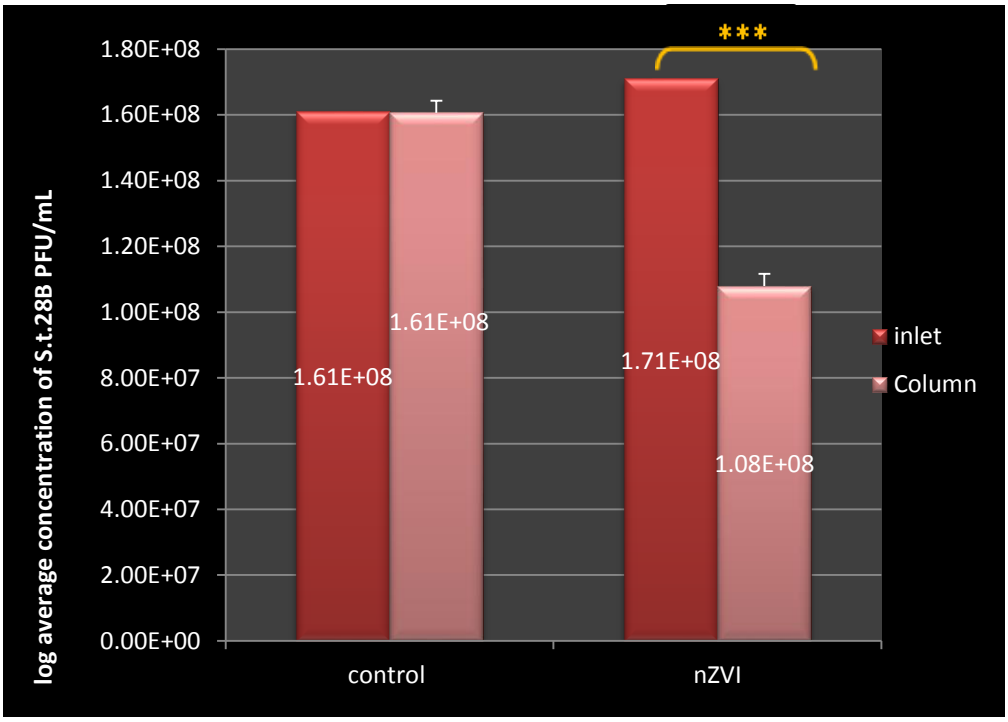


Figure9. Average concentration (PFU/mL) removal of S.t.28B in a log format for control and nZVI test (pH_0=7.5 or X, Retention time: X, Temperature=4-8 ºC, the medium was10 mM in Mili-Q water for both cases.)

5. Discussion:

According to the obtained data, there was a statistically significant reduction in virus concentrations of about 36.8 % in the presence of nZVI in the columns. Although this represents a less than 1 log reduction in the amount of virus, the result indicates a potential for the treatment. This was the first time that nZVI (nano zero- valent iron) has been tested for the removal of this thermotolerant and pH resistant bacteriophage S.t.28B, although the results do support previous tests on other viruses. For example nZVI has been applied to study inactivation mechanism of MS2 coliphage in aqueous solution, showing that direct interaction between MS2 and surface of nZVI led to the capsid damage of the virus (Kim, et al. 2011). Also in a similar experiment carried out by, Shi et al. (2012) it has been shown that zero-valent iron, ZVI, was useful for removing Aichi virus (AiV), the bacteriophages MS2 and ϕX174 from samples of water treatment plants. However those studies showed a much greater reduction that was observed in the present work, with up to a 5-6 log reduction attained, but that study used several sand/ZVI columns per treatment. Both the PFU and viral concentration results suggested that nZVI reduced the virus concentration by about 36.8%.

Virus removal mechanism

In general, inactivation and adsorption have been recognized as the two main mechanisms for virus removal from liquids which work together (Schijvenet al. 2000). Most Viruses are negatively charged, and to be more specific st28B, has a high negative surface charge at neutral pH. Since nZVI is considered to be positively charged at low pH (<pHzpc≅ 8) (Li et al. 2006), this should lead to virus absorption to the active filter media for further interaction. Then the resulting damage would be the result of a direct effect of interaction between virus and nZVI. As explained by Shi and et al. (2012), which tested the effect of nZVI on 4 viruses/phages, Adenovirus 41 (Ad41) was retained by all columns, with or without nZVI; hence the breakthrough curve, BTC, could not be plotted for the aforementioned virus. Attachment to sand for Ad41 has been explained by high isoelectric points of Ad41 fibers which reveal the importance of morphology and physic/chemical characteristic of each biological agent on

interaction and movement through a porous media. It might be that the reason for not having such a high reduction for S.t.28B is because of a generally lower adsorption for this virus. Although the reaction between nZVI and organic or inorganic contamination is relatively well understood and the chemical reactions under aerobic and anaerobic conditions have been described by several researchers, the removal and inactivation mechanism for viruses is still not completely understood (Ryan et al. 2002).

Removal of other compounds involves first a reaction starting on the surface of nZVI followed by transfer of electrons to the surface of absorbed molecule to nZVI (Li et al. 2006). Along similar lines, it has been suggested that virus adsorption to the iron oxides is as an effect of electrostatic interaction (Ryan et al. 2002) and as a result, a strong attachment and physical disintegration of the virus particle would expect to occur (Chu et al. 2001). Since the morphology of the virus particle will influence the degree of attachment, then the efficiency of virus removal will be dependent on the individual virus.

Possible nZVI remediation involving mechanisms

ZVI (Fe2+/Fe) has a relatively low standard reduction potential (E◦) of −0.44 V as compared to other metals such as Pb, Cd, Ni, and Cr, as well as many organic compounds like chlorinated hydrocarbons (Li, X.-q., et al 2006). Therefore the electron flow is usually from nZVI towards other metals or organic compounds, hence nZVI is considered to be a reductant substance.
As mentioned previously, the reactions start on the surface of nZVI, followed by a transfer of electrons to the surface of the absorbed molecule from nZVI (Li, X.-q., et al. 2006).
So far, the main applications of ZVI are based on electron donation.

$$Fe^{0} \rightarrow Fe^{2+} + 2e^{-}$$

In presence of oxygen, nZVI produce hydrogen peroxide and ferrous ion (Fe (II)) which is known as Fenton's reagent. In the next step, those reagents produce hydroxyl radical, •OH, and ferry ion, Fe (IV), through Fenton reaction. It has been shown that hydroxyl radical and ferry ion (e.g., $FeO2^{+}$) are dominant agents for inactivation of microorganism under neutral conditions. (Cundy et al. 2008; Kim et al. 2010; Nieto-Juarez et al. 2010).

$Fe^0 + O_2 + 2H^+ \rightarrow Fe\ (II) + H_2O_2$

$Fe^0 + H_2O_2 + 2H^+ \rightarrow Fe\ (II) + 2H_2O$

$Fe\ (II) + H_2O_2 \rightarrow Fe\ (III) + \bullet OH + OH^-$

$Fe\ (II) + H_2O_2 \rightarrow Fe\ (IV) + H_2O$

$Fe\ (II) + O_2 \rightarrow Fe\ (III) + \bullet O_2^-$

$Fe\ (II) + \bullet O_2^- + 2H^+ \rightarrow Fe\ (III) + H_2O_2$ (Kim et al. 2011)

The effect of Fenton reagent has been tested specifically on MS2 phage inactivation by Kim et al. (2010).A combination of Fe (II) and H2O2 (Fenton reagent) had a drastic effect on MS2 inactivation, up to a 4 log within 1 hour. But the inactivation rate was lessthan a 0.1 log for H_2O_2, and <1.5 for Fe (II) when they were used separately. The aforementioned explanation may apply for inactivation of St28B by nZVI, since the system was not deaerated although it was water saturated. A combination of Fe (II) and H2O2 (Fenton reagent) had a drastic effect on MS2 inactivation, up to a 4 log within 1 hour. Furthermore the precipitation, adsorption and transformation described as the main mechanisms involved in removing contaminants from ground water by ZVI might also apply to viruses (Li, X. 2006). (See Figure10)

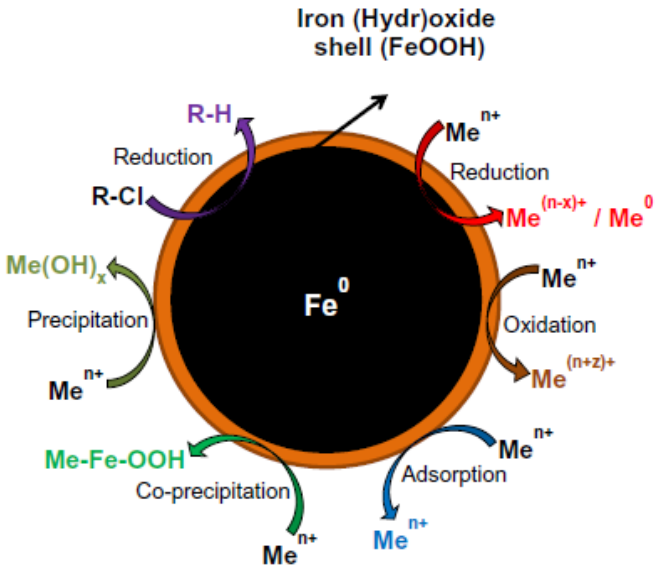


Figure 10. Expected involving mechanism on Core–shell structure of nZVI (O'Carroll et al. 2013).

Direct and indirect effect

St.28.B phage removal by nZVI could be divided into two categories: direct and indirect effect by nZVI (see Figure 11). Direct effect refers to chemical and physical interaction between microorganisms (St.28B) (Kim et al. 2010). In presence of nZVI, pH would increases in the aquatic ambient. It is assumed that partial virus removal is due to direct impact of pH increasing.

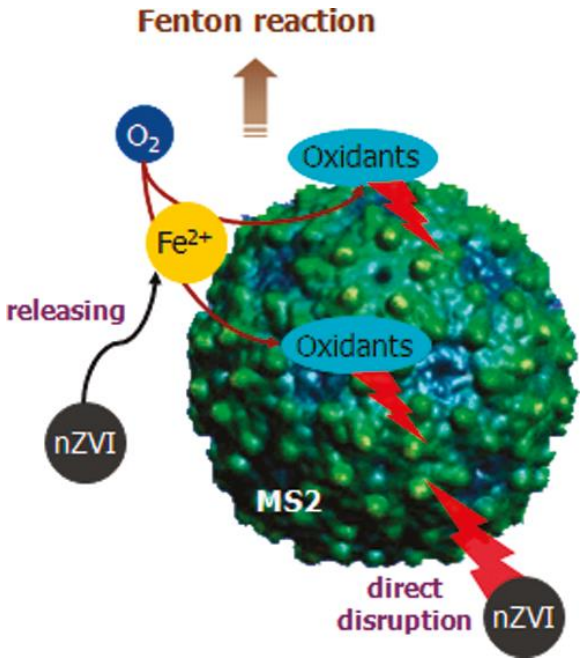


Figure 11, Schematic picture for involving mechanism for virus removal (MS2) by nZVI.

Suggestions for optimizing the System

O2 presence: Kim et al. (2011) showed that oxygen had an important effect through Fenton reaction on virus inactivation for various forms of iron, including nZVI. Thus to have a higher removal we could consider switching the condition from water saturated to unsaturated with higher amount of oxygen.

Contact time of virus with reactive media, nZVI, also plays a role on removal efficiency. Kim et al. (2011) have improved MS2 phage inactivation in their experiment from a 1.5 log with 5 min contact time to a 5.3 log reduction within 30 min. Therefore, by increasing the contact time we expect to reach higher removal rate as shown and confirmed by previous study.

nZVI concentration: Another effective factor which would enable us to have a higher removal rate is the concentration of nZVI, which in our experiment was 15 % . Kim et al. (2011) have also studied MS2 inactivation as a function of concentration within 60 min. They increased the concentration of nZVI from 0.09 to 0.9 mM and observed improved virus inactivation from less than 1 log to a 5.3 and a 2.6 log reduction at time 30 min, under air saturated and deaerated conditions, respectively.

5. Conclusions

The results conclude that nZVI mixed with glass beads was effective in removing of *Salmonella typhimurium phage 28 B* from the contaminated water. Although decreases of only 36.85% were achieved in the present set-up, optimization of the viral removal efficiency should be possible by modification of the system. nZVI has the potential to be applied as a virucidal in water treatment plants without the need for advanced infrastructure, and thus could be a relatively cheap was of purification in many parts of the world. Since nZVI has applications to a wide range of contaminants, it should be possible to meet different goals by using nZVI for water treatment. As nZV is already recognized as a versatile remediation material for a number of organic and inorganic contaminants, adding viral removal gives the potential to meet several goals simultaneously in the target system.

However, given the general concerns about potential toxicity of nanomaterials, prior to expanding the utilization and application field by switching to nZVI from routine remediation material, it would be necessary to check the effect of nZVI extensively on human health and ecosystem.

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7. Appendix

Inlet, nZVI test				
Number	PFU1	PFU2	Mean	C0
5	871	880	875.5	1.75E+08
6	85	86	85.5	1.71E+08
7	9	8	8.5	1.70E+08
8	1	1	1	2.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00


Microsoft Office Excel Worksheet

Inlet, Control test				
Number	PFU1	PFU2	Mean	C0
5	817	807	812	1.62E+08
6	80	81	80.5	1.61E+08
7	9	8	8.5	1.70E+08
8	1	1	1	2.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00

Column 1 , Control test				
Number	PFU1	PFU2	Mean	C0
5	813	802	807.5	1.62E+08
6	85	79	82	1.64E+08
7	8	8	8	1.60E+08
8	1	1	1	2.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00

Column 2 ,Control test				
Number	PFU1	PFU2	Mean	C0
5	818	791	804.5	1.61E+08
6	78	82	80	1.60E+08
7	8	9	8.5	1.70E+08
8	1	2	1.5	3.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00

Column3 ,Control test				
Number	PFU1	PFU2	Mean	C0
5	795	811	803	1.61E+08
6	78	80	79	1.58E+08
7	9	9	9	1.80E+08
8	1	1	1	2.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00

Column 1, nZVI test				
Number	PFU1	PFU2	Mean	C0
5	544	536	540	1.08E+08
6	55	52	53.5	1.07E+08
7	6	5	5.5	1.10E+08
8	1	0	0.5	1.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00

column 2, nZVI test				
number	PFU1	PFU2	MEAN	C0
5	543	552	547.5	1.10E+08
6	53	59	56	1.12E+08
7	5	6	6	1.20E+08
8	0	1	0.5	1.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00

column 3, nZVI test				
Number o	PFU1	PFU2	Mean	C0
5	524	531	527.5	1.06E+08
6	48	57	52.5	1.05E+08
7	5	5	5	1.00E+08
8	1	0	0.5	1.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0

Virus concentration immidately after Propogation Stock 1				
Number	PFU1	PFU2	Mean	C0
5	Too much	Too much	-	-
6	Too much	Too much	-	-
7	Too much	Too much	-	-
8	82	88	85	1.70E+10
9	11	10	10.5	2.10E+10
10	2	0	1	2.00E+10

Microsoft Office Excel Worksheet

Virus Concentration after storage Stock 1				
Number	PFU1	PFU2	Mean	C0
5	Too much	Too much	-	-
6	629	673	651	1.30E+09
7	91	85	88	1.76E+09
8	9	7	8	1.60E+09
9	1	1	1	2.00E+09
10	0	0	0	0.00E+00

Virus concentration immidately after propogation Stock 2				
Number	PFU1	PFU1	Mean	C0
5	Too much	Too much	-	-
6	Too much	Too much	-	-
7	102	122	112	2.24E+09
8	9	11	10	2.00E+09
9	1	2	1.5	3.00E+09
10	0	1	0.5	1.00E+10

Virus concentration after storage Stock 2				
Number	PFU1	PFU2	Mean	C0
5	Too much	Too much	-	-
6	120	125	122.5	2.45E+08
7	12	12	12	2.40E+08
8	1	1	1	2.00E+08
9	0	0	0	0.00E+00
10	0	0	0	0.00E+00

Base on Concentration :

dilutions	Inlet	Colu. 1	Colu. 2	Colu. 3
1.0E-05	1.62E+08	1.62E+08	1.61E+08	1.61E+08
1.0E-06	1.61E+08	1.64E+08	1.60E+08	1.58E+08
1.0E-07	1.70E+08	1.60E+08	1.70E+08	1.80E+08
1.0E-08	2.00E+08	2.00E+08	3.00E+08	2.00E+08
1.0E-09	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1.0E-10	0.00E+00	0.00E+00	0.00E+00	0.00E+00

t.test

p value <0.05 consider to be significant

	Inlet	Inlet	Colu. 1	Colu. 2	Colu. 3	*p* value	
1.0E-05	1.62E+08	1.62E+08	1.62E+08	1.61E+08	1.61E+08	#NAME?	no significant diff. btw Inlet and Colu.
1.0E-06	1.61E+08	1.61E+08	1.64E+08	1.60E+08	1.58E+08	#NAME?	no significant diff. btw Inlet and Colu.
1.0E-07	1.70E+08	1.70E+08	1.60E+08	1.70E+08	1.80E+08	#NAME?	no significant diff. btw Inlet and Colu.
1.0E-08	2.00E+08	2.00E+08	2.00E+08	3.00E+08	2.00E+08	#NAME?	no significant diff. btw Inlet and Colu.
1.0E-09	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	#NAME?	
1.0E-10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	#NAME?	

	Inlet		Column 1		Column 2		Column 3		*p* value
dilutions	PFU1	PFU2	PFU1	PFU2	PFU1	PFU2	PFU1	PFU2	
1.0E-05	817	807	813	802	818	791	795	811	#NAME?
1.0E-06	80	81	85	79	78	82	78	80	#NAME?
1.0E-07	9	8	8	8	8	9	9	9	#NAME?
1.0E-08	1	1	1	1	1	2	1	1	#NAME?
1.0E-09	0	0	0	0	0	0	0	0	#NAME?
1.0E-10	0	0	0	0	0	0	0	0	#NAME?

Average

	Inlet	Colu.	STD Inlet	STD Colu.
1.0E-05	812.00	805.00	-7.07E+00	1.07E+01
1.0E-06	80.50	80.33	-7.07E-01	2.73E+00
1.0E-07	8.50	8.50	-7.07E-01	5.48E-01
1.0E-08	1.00	1.17	0.00E+00	4.08E-01
1.0E-09	0.00	0.00	0.00E+00	0.00E+00
1.0E-10	0.00	0.00	0.00E+00	0.00E+00

	Inlet	Avg. Colu.	STD
1.0E-05	1.62E+08	1.61E+08	4.58E+05
1.0E-06	1.61E+08	1.61E+08	3.06E+06
1.0E-07	1.70E+08	1.70E+08	1.00E+07
1.0E-08	2.00E+08	2.33E+08	5.77E+07
1.0E-09	0.00E+00	0.00E+00	0.00E+00
1.0E-10	0.00E+00	0.00E+00	0.00E+00

Microsoft Office
Excel Worksheet

Calculation for nZVItreatment:

Base on Concentration :

	dilutions	Inlet	Colu. 1	Colu. 2	Colu. 3
	1.0E-05	1.75E+08	1.08E+08	1.10E+08	1.06E+08
	1.0E-06	1.71E+08	1.07E+08	1.12E+08	1.05E+08
	1.0E-07	1.70E+08	1.10E+08	1.20E+08	1.00E+08
	1.0E-08	2.00E+08	1.00E+08	1.00E+08	1.00E+08
	1.0E-09	0.00E+00	0.00E+00	0.00E+00	0.00E+00
	1.0E-10	0.00E+00	0.00E+00	0.00E+00	0.00E+00

***p* value < 0.05 significant**		p<0.05 *	p< 0.01 **	p<0.001 ***		
	Inlet	**Inlet**	**Colu. 1**	**Colu. 2**	**Colu. 3**	***p* value**	
1.0E-05	1.751E+08	1.75E+08	1.08E+08	1.10E+08	1.06E+08	**#NAME?**	***
1.0E-06	1.710E+08	1.71E+08	1.07E+08	1.12E+08	1.05E+08	**#NAME?**	***
1.0E-07	1.700E+08	1.70E+08	1.10E+08	1.20E+08	1.00E+08	**#NAME?**	***
1.0E-08	2.000E+08	2.00E+08	1.00E+08	1.00E+08	1.00E+08	**#NAME?**	
1.0E-09	0.000E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	**#NAME?**	
1.0E-10	0.000E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	**#NAME?**	

	Inlet	Avg. Colu.	STD Colu	STD Inlet
1.0E-05	1.75E+08	1.08E+08	2.02E+06	0
1.0E-06	1.71E+08	1.08E+08	3.61E+06	0
1.0E-07	1.70E+08	1.10E+08	1.00E+07	0
1.0E-08	2.00E+08	1.00E+08	0.00E+00	0
1.0E-09	0.00E+00	0.00E+00	0.00E+00	0
1.0E-10	0.00E+00	0.00E+00	0.00E+00	0

	Inlet		Column 1		Column 2		Column 3		*p* value	
dilutions	PFU1	PFU2	PFU1	PFU2	PFU1	PFU2	PFU1	PFU2		
1.0E-05	**871**	**880**	**544**	**536**	**543**	**552**	**524**	**531**	#NAME?	***
1.0E-06	**85**	**86**	**55**	**52**	**53**	**59**	**48**	**57**	#NAME?	***
1.0E-07	**9**	**8**	**6**	**5**	**5**	**6**	**5**	**5**	#NAME?	***
1.0E-08	**1**	**1**	**1**	**0**	**0**	**1**	**1**	**0**	#NAME?	
1.0E-09	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	#NAME?	
1.0E-10	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	#NAME?	

	Inlet	Colu.	STD Inlet	STD Colu.
1.0E-05	**875.50**	**538.33**	6.36E+00	1.01E+01
1.0E-06	**85.50**	**54.00**	7.07E-01	3.90E+00
1.0E-07	**8.50**	**5.33**	7.07E-01	5.16E-01
1.0E-08	**1.00**	**0.50**	0.00E+00	5.48E-01
1.0E-09	**0.00**	**0.00**	0.00E+00	0.00E+00
1.0E-10	**0.00**	**0.00**	0.00E+00	0.00E+00

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