The mechanism for bacteriophage f2 removal by nanoscale zero-valent iron

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A R T I C L E   I N F O
Article history:
Received 15 June 2016
Received in revised form 13 September 2016
Accepted 15 September 2016
Available online 16 September 2016

Keywords:
Nanoscale zero-valent iron (NZVI)
Virus
Bacteriophage f2
Oxygen
Mechanism

A B S T R A C T
Nanoscale zero-valent iron (NZVI) has shown excellent performance for pathogenic microorganism removal but the inactivation mechanism has not been understood clearly enough. In this study, the bacteriophage f2 removal by NZVI under aerobic and anaerobic conditions was investigated, and various factors involved in f2 removal were analyzed in detail, including the ion products of NZVI (Fe(II), Fe(III)), solid phase products, the reactive oxygen species (ROS), O2 and H⁺. In addition, the morphologies of bacteriophage f2 during reaction were observed. The results showed that the removal efficiency of bacteriophage f2 was much higher under aerobic conditions than that in anaerobic systems, and oxygen and pH were determinants for f2 removal. The oxidation of Fe(II) was a fundamental step and played a significant role in bacteriophage f2 during reaction were observed. The results showed that the removal efficiency of bacteriophage f2 was much higher under aerobic conditions than that in anaerobic systems, and oxygen and pH were determinants for f2 removal. The oxidation of Fe(II) was a fundamental step and played a significant role in bacteriophage f2 during reaction. In the presence of oxygen, the virus removal was attributed to the generation of ROS (namely -OH and -O₂⁻/C0) and the oxidized iron, in which the ROS (-OH and -O₂⁻/C0) made a predominant contribution. And the adsorption of iron oxide was responsible for the removal in oxygen depleted circumstance. In the anaerobic system, the virus removal was mainly attributed to the interaction between NZVI and bacteriophage f2. Besides, from the perspective of TEM images, the virus removal was mainly attributed to the damage of infective ability by NZVI at the initial stage of reaction, and later the virus was inactivated by the ROS generated.

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1. Introduction
Microorganism contamination in surface water and drinking water, especially some strong pathogenic and infectious viruses, can pose a serious risk and threat to public health through indirect or direct contacting with humans (World Health Organization, 2003). How to effectively inactivate these pathogenic microorganisms has been one of hot topics in the field of environmental science and engineering.

Recently, zero-valent iron has been widely used for removal of contaminants including bacteria and virus (You et al., 2005; Auffan et al., 2008; Lee et al., 2008a; Diao and Yao, 2009; Shi et al., 2012), as well as chemical pollutants. And excellent performance can be achieved by nanoscale zero-valent iron (NZVI) in the process. In addition, the microorganism removal mechanisms were discussed to some extent. However, it’s reported that the removal mechanisms of bacteria and virus were totally different. According to the published researches, the removal efficiency for E. coli was higher under anaerobic conditions than that under aerobic conditions, indicating the mechanism was mainly attributed to the direct interaction between NZVI and bacteria (Lee et al., 2008b; Kim et al., 2010b). Contrary to E. coli, the inactivation efficiency of MS2 by NZVI was much higher in the presence of oxygen, suggesting the reactive oxygen species (ROS) generated during the oxidation of NZVI was responsible for the virus removal (Kim et al., 2011).

In general, the mechanism for microorganism inactivation with NZVI has not been understood clearly enough. And the correlational studies mainly focused on E. coli and MS2, and more species should be paid attention to obtain a more generalized and elemental law. Bacteriophage f2 is an icosahedral, positive-sense single-stranded RNA virus that infects the bacterium E. coli. The pl of bacteriophage f2 is 4.0 as published (Michen and Graule, 2010). It is extremely similar to bacteriophage MS2. In China, bacteriophage f2 is usually selected as surrogates for human enteric...
viruses due to its characteristics resemblance to many human enteric viruses and its ease of use. Although the mechanism of MS2 inactivation by NZVI has been studied and proposed, the study on removal of bacteriophage f2 with NZVI is still limited. In our previous work, we have studied the performance of bacteriophage f2 removal by NZVI (Cheng et al., 2014). And in this study, oxygen, iron ion, ROS, and NZVI residue, all of the relative factors were taken into account to get a comprehensive understand of the mechanism for f2 removal by NZVI. In addition, the transformation of NZVI and f2 were examined through instrumental analysis, which further verified the interaction between NZVI and f2.

2. Materials and methods

2.1. Chemicals

The chemicals used in this study were of reagent grade and purchased from the Sinopharm Chemical Reagent Beijing (Beijing, China), including NaBH₄, FeSO₄, HCl, NaOH, NaCl, nutrient agar medium, nutrient broth, agar, dimethyl sulfoxide (DMSO), and isopropanol. Superoxide dismutase (SOD) was purchased from Sigma Company. All chemicals were used without further purification. And all solutions were prepared with ultrapure water from a Milli Q system (Millipore, US).

2.2. Preparation of NZVI

NZVI particles were synthesized by aqueous-phase reduction of ferrous sulfate with sodium borohydride via following chemical reaction:

\[
2\text{Fe}^{2+} + \text{BH}_4^- + 3\text{H}_2\text{O} \rightarrow 2\text{Fe}^{0} + \text{BO}_3^{3-} + 6\text{H}^+ + 2\text{H}_2
\]

Briefly, aqueous solution of sodium borohydride was added dropwise to aqueous solution of ferrous sulfate with a final molar ratio of more than 2:1 (sodium borohydride to ferrous sulfate). The solutions were bubbled with argon to remove dissolve oxygen throughout the whole process. Then the products were separated from the mixture through natural sedimentation, rinsed three times with deoxygenated water, and vacuum dried.

2.3. Bacteriophage f2 preparation and plaque forming unit (PFU) assay

Bacteriophage f2 and its host bacteria (E. coli 285) were purchased from institute of Hygiene and Environmental Medicine, Academy of Military Medical Sciences, China. Bacteriophage f2 was duplicated and purified as described in our previous study (Cheng et al., 2014). Bacteriophage f2 was incubated with the double agar layer method and the concentration was reported as plaque-forming units per milliliter (PFU/mL). Only the plates ranged from 30 to 300 plaques were valid. All the virus tests were performed in triplication.

2.4. Experimental procedure

The batch experiments were performed at 25 °C using 1000 mL four-necked flask containing 500 mL virus suspension. The initial concentration of bacteriophage f2 was 10⁴ PFU/mL. For the tests under aerobic conditions, the experiments were performed exposing to the air. For anaerobic circumstance, the solution was bubbled with ultrapure Ar through a tube until no oxygen was detected in the system. Then the tube was lifted above the solution surface, and Ar was kept flowing, ensuring the anaerobic condition but little turbulence to the solution. NZVI was added in the solution and then the flasks were put into a shaking table with a rotate speed of 100 rpm. Samples of 1 mL were withdrawn from various test groups at predetermined time intervals and each group was offset with injecting 1 mL ID water. Then the samples were incubated in incubator at 37 °C and analyzed immediately for viable virus concentration by the plaque assay. The removal efficiency of virus was calculated as “log(N/N₀)”, where “N” and “N₀” referred to the concentration of virus in the solution at t and the initial time, respectively.

Hydrochloric acid and sodium hydroxide solutions were used to adjust the initial pH values of solutions. The systems without NZVI were used as controls.

For the quenching study, Dimethyl sulfoxide (DMSO) was used as a hydroxyl radical (-OH) and ferryl ion (Fe(IV)) scavenger, isopropanol was used as a hydroxyl radical (-OH) scavenger, and superoxide dismutase (SOD) was used as a superoxide radical (-O₂) scavenger (Kim et al., 2010a; Katsyriannis et al., 2008; Keenan and Sedlik, 2008). The dosage used in the study was 50 mM DMSO, 50 mM isopropanol, and 4 U/mL SOD, respectively. And the systems with phage f2 and scavenger were used as controls.

2.5. Characterization and tracking of iron particles

Morphology of the synthesized NZVI particles were observed with a scanning electron microscope (SEM, Model JSM-6301F by JEOL) equipped with energy dispersive spectroscopy (EDS). The as-prepared materials were spheroidal particles with a size of about 50 nm, and the SEM image was shown in Supporting Materials.

XRD patterns of as-prepared NZVI and the solid residue after reaction were recorded with a Rigaku D/max-RR X-ray diffractometer with Cu Kα radiation (λ = 0.1542 nm). A counting time of 25 s per 0.05° step was used for the 2θ range 10–90°.

The ions that NZVI released to the solutions were determined with o-phenanthroline spectrophotometric method (Kim et al., 2011).

2.6. Characterization of bacteriophage f2

The samples of bacteriophage f2 and the solutions with bacteriophage f2 that reacted with NZVI for different periods were investigated using a transmission electron microscope (TEM, JEM-1400, JEOL, Tokyo, Japan) by conventional negative staining. To protect the structure of bacteriophage f2 from damaging, a few drops of fixative were added to the samples and allowed to set for 20 min. The samples were applied to holey carbon-coated, 300-mesh, copper grids.

3. Results and discussion

3.1. Bacteriophage f2 removal by NZVI under anaerobic/aerobic conditions

Control tests without NZVI indicated that little inactivation happened to bacteriophage f2 under both of anaerobic and aerobic conditions during experimental time. As shown in Fig. 1, oxygen demonstrated a significant effect on the removal of bacteriophage f2. In the absence of oxygen, the reaction reached balance within 5 min when the NZVI dosage was 0.2 mM. And no more changes were observed as the reaction time extended to 60 min, resulting in a removal efficiency of 0.7 log. While under aerobic conditions (DO: 7.82 mg L⁻¹), the removal efficiency of 3.2 log could be attained after 60-min reaction. Obviously, the removal efficiency was much higher in aerobic systems than that in anaerobic ones. The process was mainly related to the oxidation of zero-valent iron, which improved the removal efficiency of contaminants (Wang et al.,...
In addition, the removal rate went up as the dosage of NZVI increased no matter under anaerobic or aerobic conditions. When the dosage of NZVI was 0.5 mM, the removal efficiency was 2.4 log and 4.1 log after 60-min reaction in the anaerobic and aerobic system, respectively. As far as the removal efficiency is concerned, the adsorption effect of NZVI can make a certain contribution due to its high specific surface and high surface reactivity (Nurmi et al., 2005; Cheng et al., 2010). Apart from the role of NZVI alone in the systems, it may involve generation of various by-products through the oxidation of NZVI. During the experiments, part of iron existed as ions in the solution including Fe(II) and Fe(III), and part of iron existed as solid residue at the bottom of flask. Then the ions in the solution and the solid residue were detected and their effects were studied.

3.2. The iron released into the system

The dissolved iron released from zero-valent iron into solution in anaerobic/aerobic systems was detected and the results were presented in Fig. 2. In the absence of oxygen, ferrous was dominating, approximately accounted for 70% of the total dissolved iron (Fig 2a), whereas ferric was the main product in the presence of oxygen (Fig 2b). The presence of ferrous ion and ferric ion presumably were the initial factors when the removal curves were taken into consideration.

As reported, the removal of bacteriophage f2 by NZVI involved the generation of intermediates, such as Fe^{2+}, Fe^{3+}, reactive oxygen species (Fe(IV), ·OH and ·O2), originated from the Fe-participated reactions (Lee et al., 2008b; Kim et al., 2011). In order to
investigate which one of the intermediates played a dominant role in the bacteriophage f2 removal, a series of experiments were carried out as follows.

3.3. Bacteriophage f2 removal by Fe(II)/Fe(III) under anaerobic/aerobic conditions

As shown in Fig. 3a, the removal efficiency reached balance within 10 min under anaerobic conditions and resulted in a removal efficiency of approximately 1.1 log and 2.5 log for 0.2 mM and 0.5 mM Fe(II) system, respectively. However, no viable bacteriophage f2 was detected after 30-min reaction in both of the two systems with different NZVI dosage when the systems were exposed to the air, suggesting that the removal process was oxygen dependent. Comparing the results in Figs. 1 and 3a, Fe(II) was much stronger than NZVI in bacteriophage f2 removal under aerobic conditions. Then it can be concluded that the oxidation of Fe(II) was the fundamental step in terms with f2 removal in the study.

For Fe(III), the removal efficiency of phage f2 can be neglected and there was little difference between anaerobic and aerobic systems (Fig. 3b). It indicated that Fe(III) made little contribution to the removal of bacteriophage f2. It is likely that limited solubility of Fe(III) made the removal of virus be difficult under neutral pH circumstances (Lee et al., 2008b; Keenan and Sedlak, 2008).

As stated above, Fe(II) played a significant role in terms with bacteriophage f2 removal, especially in the aerobic conditions. In the Fe(II) and oxygen system, superoxide radical and hydrogen peroxide can be generated from the reaction of Fe(II) and oxygen through one-electron transfer (reactions 2 and 3) (Lee et al., 2008b; Kim et al., 2011). And hydroxyl radical was subsequently yielded through Fenton reaction (reaction 4). Hence the formation of reactive oxygen species (ROS) (e.g., $O_2^\bullet$, OH) were the predominant reason for the removal of bacteriophage f2. However, previous
studies have reclaimed that a less reactive oxidant than \( \cdot OH \), most likely Fe (IV) (reaction 5) accounts for the removal at neutral pH values (Hug and Leupin, 2003; Keenan and Sedlak, 2008; Katsoyiannis et al., 2008). The effect of various ROS on the removal will be discussed in the subsequent sections.

\[
\begin{align*}
\text{Fe}^{(II)} + O_2 & \rightarrow \text{Fe}^{(III)} + \cdot O_2 \\
\text{Fe}^{(II)} + \cdot O_2 + 2H^+ & \rightarrow \text{Fe}^{(III)} + H_2O_2 \\
\text{Fe}^{(II)} + H_2O_2 & \rightarrow \text{Fe}^{(III)} + \cdot OH + OH^- \\
\text{Fe}^{(II)} + H_2O_2 & \rightarrow \text{Fe}^{(IV)} + H_2O
\end{align*}
\]

3.4. Bacteriophage f2 removal by NZVI under different pH values

The effect of pH on bacteriophage f2 removal was investigated and the results were presented in Fig. 4. Similar conclusion was obtained that the removal efficiency was much higher under aerobic conditions (Fig. 4b) than that in anaerobic systems (Fig. 4a). Besides, the removal efficiency was increased with the decreasing of pH value in anaerobic or aerobic systems. The results indicated that pH and oxygen were determined factors for f2 removal, which was primarily associated with the extent of corrosion and oxidation of NZVI. In alkaline conditions, the activity of NZVI was easily reduced due to the formation of iron hydroxide coating on the surface of NZVI, while the passivation layer can be removed and NZVI can still keep a strong activity in acid conditions. In addition, the corrosion rate was accelerated in acid systems, especially under aerobic conditions, resulting in generation of more ions and ROS (reaction 6 and reaction 4).

\[
\begin{align*}
\text{Fe} + O_2 + 2H^+ & \rightarrow \text{Fe}^{(II)} + H_2O_2
\end{align*}
\]

3.5. The transformation of NZVI

To further investigate the transformation of NZVI, XRD was...
applied to examining the component of the solid residue after reaction as presented in Fig. 5. The results indicated that the final products were totally different under aerobic and anaerobic conditions. NZVI was instantaneously oxidized and converted into oxidation compounds in the presence of oxygen in water. The strong peaks at 14.1°, 26.8°, 46.8° are consistent with the crystallographic lepidocrocite (γ-FeOOH) and the peak at 36.2° refers to magnetite (Fe₃O₄) and/or maghemite (Fe₂O₃) because they are isomorphous and indistinguishable (Fig. 5b). The results were similar to previous study (Noubactep, 2010). And the Fe(II)/Fe(III) and Fe (III) corrosion products indicated that Fe (II) formation was an intermediate step in the transformation process.

In the absence of oxygen, the XRD analysis suggested that the residue was almost zero-valent iron (Fig. 5c). Considering the ions released into the solution determined above, it can be concluded that NZVI and Fe(II) were co-existed in the anaerobic systems. As a result, the removal of bacteriophage f2 was the combined effect of NZVI and Fe(II) under anaerobic conditions. However, the yield of Fe(II) in the system (Fig. 2) was so low (10−20 μM) that the removal by Fe(II) was limited. Therefore, it can be explained that the removal of virus was mainly attributed to the interaction between NZVI and bacteriophage f2 in anaerobic systems.

3.6. The bacteriophage f2 removal by NZVI residue

To examine the effect of NZVI residue on bacteriophage f2 removal, a test was performed using the NZVI residue from the aerobic system. And the results were illustrated in Fig. 6. It turned out that the NZVI residue still had a wonderful ability with respect to the removal of virus, resulting in a removal efficiency of approximately 3.0 log after 60-min reaction. Based on the results of XRD in Fig. 5, the NZVI residue was composed of γ-FeOOH and Fe₂O₄ and/or Fe₂O₃, which were of excellent abilities for removing contaminants (Bradley et al., 2011; Singh et al., 2011). As mentioned above, the pI of bacteriophage f2 is 4.0, and the phage f2 was electronegative in the system. As a result, virus could be adsorbed on the surface of iron oxide due to its great electrostatic force (Ryan et al., 2002; Gutierrez et al., 2009). Then the removal of bacteriophage f2 can be attributed to the combined adsorption effect of γ-FeOOH and Fe₂O₄ and/or Fe₂O₃ after the total oxidation of NZVI.

3.7. The morphological changes of bacteriophage f2

The morphological changes of bacteriophage f2 were investigated in this study, as well as NZVI and its products. As shown in Fig. 7a, bacteriophage f2 is a six-sided shape structure with a little tail (actually, f2 is an icosahedron structure in three-dimension) under electron microscope, and the measured diameter ranges from 25 to 30 nm. After reaction for 10 min, f2 was adsorbed onto the surface of NZVI, in which aggregation happened, suggesting a brilliant affinity of NZVI for f2. The shape of f2 began to change into irregular structure slowly but relatively intact on the adsorbed sites. In addition, aggregation could also occur in the solution (Fig. 7b). As the reaction continued, the outer structure of f2 broken down and mutually fused with each other, finally larger pieces of structure formed (Fig. 7c). The damaged f2 coating on the surface of NZVI suggested that a proportion of f2 removal was attributed to the direct interaction between NZVI and f2 bacteriophage (Fig. 7d and e).

From the perspective of TEM images, the virus inactivation process was proposed. At the initial stage of reaction, no fatal damage happened to bacteriophage f2 due to short contacting with NZVI and limited yield of ROS, only slightly structural lesion occurred, e.g., the damage of infective tail, which was responsible for injecting the RNA into E. coli host. However, at the later stage of reaction, a significant portion of bacteriophage f2 was inactivated as the reaction time extended and the ROS generated increased.

3.8. The bacteriophage f2 removal by NZVI in the presence of ROS scavengers

The ROS generated in the system was responsible for the removal of contaminants under aerobic condition based on the above analysis, as well as previous reports (Pignatello et al., 2006; Diao and Yao, 2009; Liga et al., 2011; Robertson et al., 2012). In this study, a series of experiments were carried out to determine the ROS in solution. However, the ROS are transient and difficult to be detected directly. So an indirect method was applied to reflecting the roles of the ROS in bacteriophage f2 removal by adding radical scavengers into systems (Zhang et al., 2008; Li et al., 2009; Kim et al., 2010a). Dimethyl sulfoxide (DMSO) was used as a hydroxyl radical (·OH) scavenger, isopropanol was used as a hydroxyl radical (·OH) scavenger, and superoxide dismutase (SOD) was used as a superoxide radical (·O₂) scavenger. The control experiments with phage f2 and scavenger confirmed that little inactivation was occurred when the scavengers were added into the bacteriophage f2 solution without NZVI. It indicated that the scavenger itself had little effect on the bacteriophage f2.

As shown in Fig. 8, both of DMSO and isopropanol had a strong effect on the removal efficiency and the difference of removal efficiencies between the two systems was negligible, revealing that the yield of Fe(IV) was very limited or the formation of Fe(IV) did little contribution to the f2 removal. Therefore the effect was mainly attributed to the generation of hydroxyl radical (·OH) by NZVI under aerobic conditions, which was different from previous studies (Kim et al., 2011). In addition, a significant inhibition was observed in the presence of SOD, implying that ·O₂ played a key role in the f2 removal during the reaction, which was consistent with the Fe(II)-participated fundamental reaction (reaction 2).

4. Conclusions

The removal efficiency of bacteriophage f2 by NZVI was much higher under aerobic conditions than that in anaerobic systems, and the removal rate was increased as the dosage of NZVI increased.
no matter under anaerobic or aerobic conditions. From the perspective of removal mechanism, the relative factors including the ion products of NZVI (Fe(II), Fe(III)), solid phase products, the reactive oxygen species (ROS) and $H^+$ were studied, as well as oxygen. The results indicated that the oxidation of Fe(II) was a fundamental step and played a significant role in bacteriophage F2 removal, especially in the aerobic systems. In addition, the removal efficiency was increased with the decreasing of pH value in anaerobic or aerobic systems, and the removal efficiency was much higher under aerobic conditions than that in anaerobic systems under all experimental pH values (5–9), which indicated that pH and oxygen were determined factors for F2 removal. In the presence of oxygen, the virus removal was attributed to the generation of ROS (namely $OH$ and $O_2^*$) and the oxidized iron, in which the ROS ($OH$ and $O_2^*$) made a predominant contribution. And the adsorption of iron oxide was responsible for the removal in oxygen depleted circumstances. In the anaerobic systems, the virus removal was mainly attributed to the interaction between NZVI and bacteriophage F2. Meanwhile, the TEM images indicated that the virus removal was mainly attributed to the damage of infective ability by NZVI at the initial stage of reaction, and later the virus was inactivated by the ROS generated.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.09.025.

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