Effects of Nano-Scale Zero Valent Iron Fresh and Aged Particles on Environmental Microbes

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ABSTRACT

Currently, nano-scale zero valent iron particles (nZVI) are being increasingly used in many types of environmental remediation. Due to their usage, nZVI can be left in the environment and may cause toxic effects to living beings, especially surrounding microorganisms. Environmental bacteria in soil and water are some of the main factors affecting plant productivity and other microorganisms in ecosystems. This study evaluated the toxicological effects of nZVI and aged nZVI on bacteria commonly found in the environment. Bacterial, namely *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *Rhodococcus* sp., were treated with different concentrations of nZVI at different times of exposure in *in vitro* conditions, and bacterial cell viability was determined in order to analyze the toxic effects of nZVI over the course of treatments. The data revealed that at the highest nZVI concentration (1,000 mg/L), *B. subtilis* and *Rhodococcus* sp. had the highest resistance to nZVI (49.35% and 48.31% viability) and less resistance in *P. aeruginosa* (2.26%) and *E. coli* (0.50%) was observed. The growth of microorganisms significantly increased after exposure with seven and 14-day aged nZVI particles. Therefore, the toxicity of aged nZVI to microbial organisms was reduced. Hence, this study demonstrated the toxic effects of nZVI and aged nZVI particles on several species of bacteria *in vitro*. Less toxicity to bacteria was observed in aged nZVI. These findings provide more understanding in the toxic effect of nZVI to microorganisms.

1. INTRODUCTION

Nanotechnology is a rapidly growing scientific area. There are several industrial applications using nanomaterials for development of many commercial products and environmental remediation (Li et al., 2006; Pulit-Prociak et al., 2015). Since 1996, zero-valent iron nanoparticles (nZVI) have been presented as remediation material for removing environmental hazardous contaminants (Wang and Zhang, 1997; Zhang et al., 1998; Lien and Zhang, 1999). For instance, nZVI have been used for removing heavy metals such as cadmium, mercury, silver and nickel, as well as chlorinated hydrocarbons, from contaminated soil and water (Barzan et al., 2014). nZVI particles smaller than 100 nm can be successfully used in degrading chemical contaminants including organic and inorganic pollutants in wastewater and ground water (Diao and Yao, 2009).

In 2005, more than 10 trichloroethane (TCE) contaminated sites in USA were treated by using nZVI (NSCEP, 2005). Mueller et al. (2012) reported the use of nZVI to remove chlorinated hydrocarbon from contaminated sites in Germany and Czech Republic. More recently, nZVI has been used to clean many contaminated fields including both soil and groundwater (Chowdhury et al., 2015; Galdames et al., 2020). Therefore, the increasing use of nZVI can lead to their presence in the environment. The residual nZVI may come from its direct injection into the contaminant sources such as underground or in underwater sludge. After use, the reacted nZVI has been discarded in the treated areas.

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Some research shows that microorganism viability in contaminated water exposed with nZVI can be decreased, but the effect depends on many conditions such as exposure time and microorganism species (Lee et al., 2008; Chen et al., 2013; Bensaida et al., 2021). The toxicity level of nZVI is related to the composition and structure of the cell in different types of microorganisms. Gram-positive cells showed more resistance than Gram-negative cells. Some previous research also suggested that the different resistance of Gram-positive and Gram-negative bacteria to nZVI was due to the difference in their ability to fight reactive oxidative species (ROS) created by nZVI reactions with the surrounding environment. nZVI particles have been shown to be able to react with O_2 and H_2O in the environment to produce Fe(II) and H_2O_2, which continuously create hydroxyl radicals (OH·) and other ROS by Fenton reaction (Wu et al., 2014). The resistance ability was related to the metalloregulatory protein and ROS scavenger production (Chen et al., 2013). Moreover, the effect of nZVI on bacteria was not only dependent upon genus and strain but also growth phase, where the toxicity to the bacterial cells was higher in exponential and decline phases than in lag and stationary phases (Chaithawiwat et al., 2016). There are several *in vitro* reports of the antibacterial effect of nZVI and a decrease of enzyme activity in microorganisms including *Escherichia coli*, *Staphylococcus aureus*, and *Dehalococcoides* spp. (Pawlett et al., 2013). The effect of nZVI on soil microbial communities was dependent on the level of organic matter and soil mineral type. It was also found that the nanoparticles did not change the soil composition or the organic carbon in the soil extract, but affected the soil bacterial community composition (Ben-Moshe et al., 2013). These studies have confirmed the toxic effect of nZVI on surrounding microorganisms. However, nZVI has still been used due to its ability to efficiently and quickly remove many hazardous contaminants in the environment.

Due to the large areas of contamination, high amounts of nZVI (in the form of sludge) have been directly injected at the contaminant sources to create high concentrations (>1,000 mg/L) in the contamination site (Li et al., 2006; Chowdhury et al., 2015; Galdames et al., 2020). After use, a large quantity of used nZVI has been discarded in the remediation sites. Therefore, this research focused to study and compare the toxicity of both fresh nZVI and aged nZVI (used nZVI) to bacterial viability that live in natural environment in vary concentrations especially at high concentration and exposure times. The results of this study will reveal the safety and reliability of using nZVI as a material to remove several hazardous substances contaminating the environment.

2. METHODOLOGY

2.1 Chemicals and materials

Ferric chloride ($FeCl_3$) was obtained from Alfa Aesar. Sodium borohydride ($NaBH_4$) with 98% purity was used in this work. Pentane (99%), spectrophotometric grade was obtained from Sigma-Aldrich. Ethanol (95%) was purchased from Pharmco-AAper.

2.2 nZVI synthesis

The nZVI synthesis method in this research followed previous publications (Sun et al., 2006; Sun et al., 2007; Jiemvarangkul et al., 2011; Padungthon et al., 2020). Briefly, nZVI particles were manufactured by titration of $FeCl_3$ solution (1.6140 g/L $FeCl_3 \cdot 6H_2O$ dissolved in 120 mL distilled water) with $NaBH_4$ solution (0.9108 g/ L $NaBH_4$ dissolved in 120 mL distilled water) rapidly, while mixing by overhead stirrer at a stirring speed of 400 rpm. The mixing was continuously performed until the color of the mixing solution turned from yellow to black particles. The synthesis reaction is presented as follows:

$$4Fe^{3+}_{(aq)} + 3BH_4^-{}_{(aq)} + 9H_2O \rightarrow 4Fe^0_{(s)} + 3H_2BO_3^-{}_{(aq)} + 12H^+_{(aq)} + 6H_{2(g)} \quad (1)$$

At least 10 minutes of mixing time were needed to complete the reaction above. The nanoparticles were collected by vacuum filtration through 0.4 µm filter papers and were then washed by 5% ethanol solution. The average size of the particles was around 60-70 nm, as measured by transmission electron microscopy (TEM) and a combined acoustic/electro-acoustic spectrometer (Sun et al., 2006). The harvested particles were washed by 5% ethanol and kept submerged in ethanol at low temperature in a sealed polyethylene container. The moisture of contained particles was usually about 70%. nZVI solution (1,000 mg/L) was prepared by mixing 10 mg of filtered nZVI particles with 2 mL sterile water into a homogeneous solution, then adding 8 mL of sterile water, and mixing by vortex for five minutes. nZVI solution was prepared to create three conditions of 10, 100, and 1,000 mg/L. All nZVI solutions were incubated at

room temperature on a Horizontal shaker for seven days and 14 days to prepare aged nZVI.

2.3 Transmission electron microscopy (TEM)

The images of nZVI were taken by a Philips EM 400T TEM (Philips Electronics Co., Eindhoven, Netherlands) operated at 100 kV. The particle samples for TEM were set by placing three droplets of the sample suspension solution onto a holey carbon film (Ernest Fullam, Inc., Latham, NY), which was completely dried in a fume hood before taking TEM images (Sun et al., 2006).

2.4 Luria-Bertani broth (LB broth) and Luria-Bertani agar (LB agar) preparation

Luria-Bertani broth contained 10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L NaCl. The solution pH (7.0) of LB broth was measured by a pH meter and sterilized by autoclave at 121°C, 15 lbs/in^2 for 15 minutes. Luria-Bertani agar (LB agar) was prepared with 4 g LB agar powder with 1 L of distilled water at pH 7.0 and sterilized by autoclave. LB agar solution was poured in Petri dishes to form LB agar, and they were kept at 4°C if not immediately used.

2.5 Bacterial suspension and yeast suspension

Bacterial suspension dilutions for *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *Rhodococcus* sp. were prepared by inoculation of cultured bacteria from LB agar for 18-24 h, 35±2°C with 3 mL sterile water pH 7.4. The bacterial solution concentration was adjusted to 0.5 McFarland standard, then diluted into 10^7, 10^6, and 10^5 CFU/mL. For bacterial suspension dilution, yeast suspension dilutions for *C. albicans* were prepared following the same procedure with the strain cultured on Luria-Bertani agar and preparing a final concentration dilution to 10^5 CFU/mL. All microorganisms used in this research have been easily found in natural waters and environments. They were chosen to represent both Gram-positive and Gram-negative bacteria and yeast (Phopin et al., 2016).

2.6 Effects of nZVI on microbes

The bacterial dilution (10^5 CFU/mL) and yeast dilution (10^5 CFU/mL) of each strain were prepared in four conditions: (i) 1 mL of suspension without nZVI solution for control group; (ii) 1 mL of suspension with 10 mL of 10 mg/L nZVI solution; (iii) 1 mL of suspension with 10 mL of 100 mg/L nZVI solution; and (iv) 1 mL of suspension with 10 mL of 1,000 mg/L nZVI solution. Each condition, after mixing by vortex for five minutes, was transferred onto two LB agar plates, and then incubated at 35±2°C for 24 h. for *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*, but 48 h. for *Rhodococcus* sp. and *C. albicans*. Viable colony counts were determined at the end of incubation time. The treatments were compared with the control group, and the experiments were made in triplicate for accuracy. Statistical T-test analyses and One-Way Analysis of Variance (ANOVA) were conducted to compare the treatments at percent confidence of 95% (α=0.05) and *p*-values were calculated.

3. RESULTS AND DISCUSSION

3.1 nZVI properties

The color of nZVI particles sludge was black. After drying, it appeared be a black powder. The average pH of the solution containing 0.5 g of nZVI in 100 mL of deionized water was around 7.8 and the moisture of harvested nZVI was around 70%. TEM image showed a cluster of small particles with a chain-like form (Figure 1). The small individual particles had a diameter less than 100 nm (Figure 1).

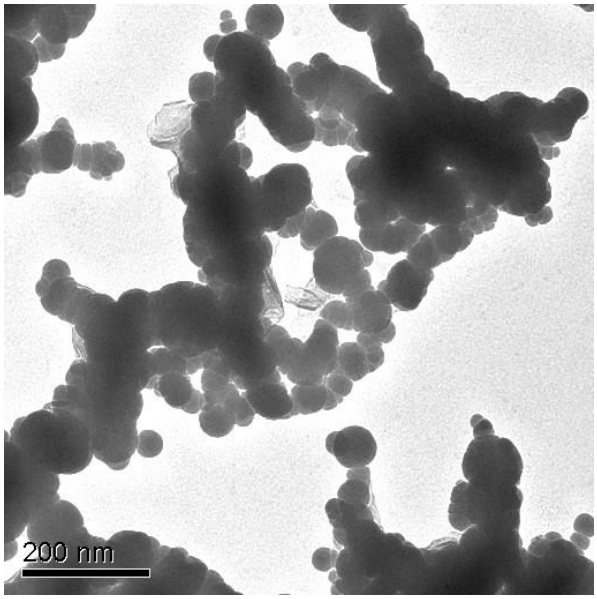

Figure 1. TEM images of nZVI particles obtained in this study.

3.2 nZVI toxicity to microorganisms

Using cytotoxicity assay to analyze the effect of nZVI on bacterial and yeast, high nZVI concentration significantly decreased microorganism viability. The results of nZVI toxicity on tested microbes are presented in Figure 2. With the high concentration of 1,000 mg/L of nZVI, the viability of *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albiacans* decreased to 0.50%, 2.26%, 7.92%, and 12.49%, respectively. *B.*

subtilis and *Rhodococcus* sp. were more resistant to toxicity with viability of 49.35% and 48.31%. Thus, the results revealed that nZVI toxicity was related to the genus or species of microbes. With nZVI concentration at 1,000 mg/L, the highest resistance was observed in the Gram-positive group (*B. Subtilis* at 49.35% and *Rhodococcus* sp. at 48.31%) and less resistance was observed in Gram-negative (*P. aeruginosa* at 2.26% and *E. coli* at 0.50%) (Figure 2).

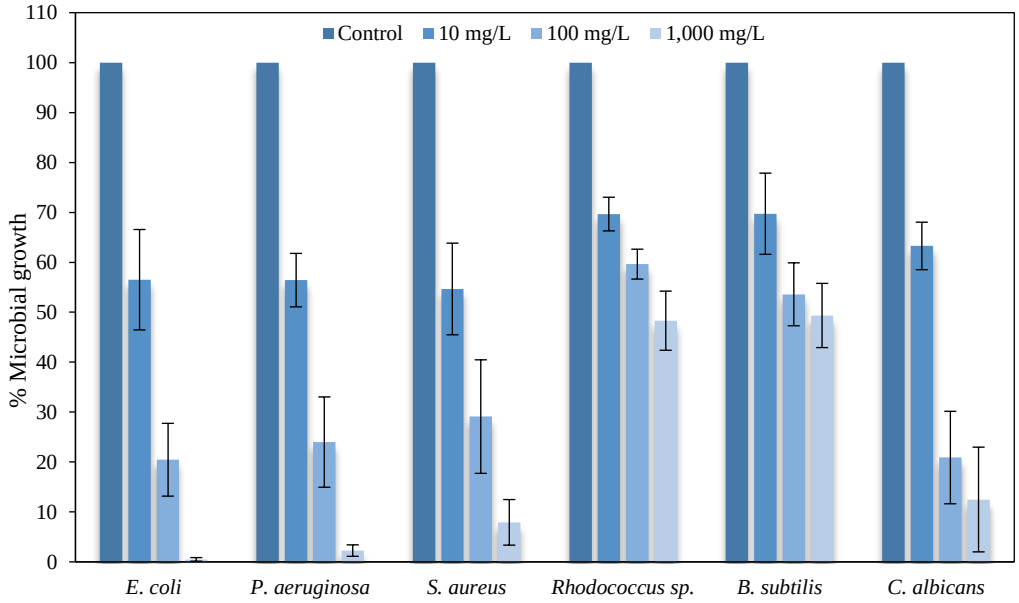

Figure 2. Percent of bacterial colony count compared to the control group after incubating with nZVI

Figure 3 shows the toxic effect of nZVI at various concentration to microorganisms. The growth of bacterial strains (except *C. albicans* as yeast) was linearized with logarithm of concentration. The correlation R^2 of all group data were higher than 0.95 indicating the toxic effect relationship with nZVI concentration. Moreover, based on Figure 3, the results showed the clear picture of the different strains of bacteria resistance to nZVI effects. The upper group of data presents Gram-positive bacteria with higher growth percent compared with the lower group which was Gram-negative. The statistical t-test of percent growth of the Gram-positive group showed a significant difference than Gram-negative bacteria with *p*-value less than 0.05 (*p*-value=0.0048).

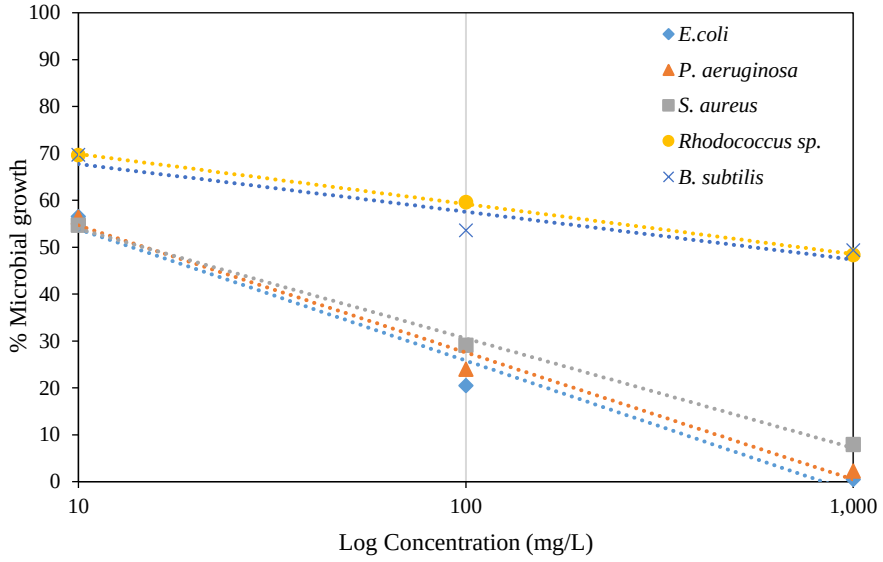

Figure 3. Bacteria strains and nZVI concentration relation with percentages of microbial growth.

The toxicity of nZVI to bacteria could be explained as a cytotoxicity mechanism related to the reactive oxidative species (ROS) production, the oxidative stress, and the liberation of toxic Fe(II), which disturbs the electronic and/or ion transport activity in the cell membrane (Kumar et al., 2017). The oxidation reaction of nZVI particles with intracellular oxygen could produce Fe(II) and hydrogen peroxide that induced oxidative stress by producing ROS (Lee et al., 2008; Kim et al., 2010; Wu et al., 2014). Diao and Yao (2009) found that nZVI particles attached to *B. subtilis* cell walls and coated their surface. Fe(II) released due to nZVI reaction may be able to enter the cytoplasm of cells and create oxidative stress, which can damage the cell membrane, leading to cell death. Cell death occurred because of DNA injuries *in vivo* and membrane damage by ROS (Sacca et al., 2014). However, the toxic effect was dependent on the bacterial species and the environmental stress level. Gram-positive and Gram-negative bacteria might have different ROS confrontation mechanisms (Chen et al., 2013). The mechanism model of nZVI particles causing toxicity to bacteria cells is proposed in Figure 4. After nZVI particles enter into the bacteria environment, they are able to cover the surface of the bacteria cell and react with oxygen in the surrounding environment to produce Fe(II) and H_2O_2, which are able to be transported into the inner cell through cell wall. Then, Fe(II) and H_2O_2 create activities to form ROS, which are harmful to bacteria.

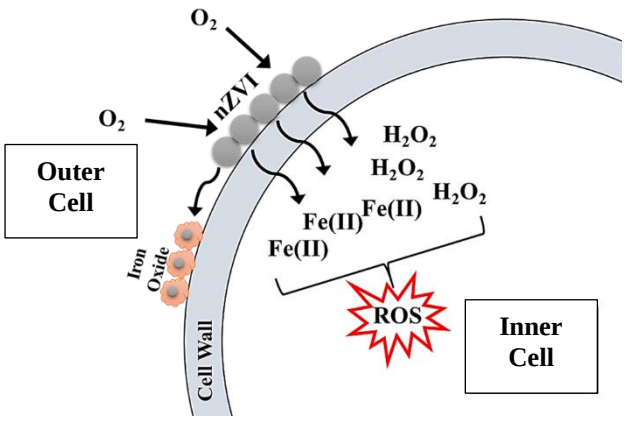

Figure 4. The proposed mechanism model of nZVI activities to cause toxicity to bacterial cell

Due to bacterial characteristics, the Gram-positive cell wall, a 20-80 nm peptidoglycan layer, is 10 times thicker than the Gram-negative cell wall, resulting in additional protection to nZVI toxicity in the Gram-positive bacteria (Silhavy et al., 2010; Chen et al., 2011). Therefore, lipoteichoic acid, a component of the cell wall of Gram-positive bacteria, might have an important role in the toxic resistance when forming a chelating complex with nZVI (Chen et al., 2013). The greater resistance of *B. subtilis* and *Rhodococcus* sp. in this study was caused by their cell wall, as well as the endospore production of *B. subtilis* and mycolic acid of *Rhodococcus* sp. *B. subtilis*, a Gram-positive bacterium with a thick cell wall has a better defense system against environmental stress. Although *B. subtilis* was treated with 1,000 mg/L of nZVI, they were still able to growth with 49.39% viability. The thicker cell wall of Gram-positive bacteria may absorb most of Fe(II) and H_2O_2 released from nZVI reaction with O_2 in solution, so they cannot enter the inner cell to produce ROS as in Figure 4. On the other hand, the lower resistance of Gram-negative bacteria such as *E. coli* may be due to their thinner cell wall that cannot block Fe(II) and H_2O_2 to go inside their cells.

Based upon the results, *C. albicans*, a yeast, was able to resist the effect of nZVI particles better than all Gram-negative bacteria, but its resistance was still less than Gram-positive bacteria, even though *C. albicans* had thicker cell wall (average around 150 nm) than Gram-positive bacteria cell wall (20-80 nm) (Plaine et al., 2008; Silhavy et al., 2010; Klis et al., 2014). The cell wall of *C. albicans* consists of two-layers, and the major structures of its cell wall are beta-glucan and chitin (Plaine et al., 2008). However, there was a discovery that the thickness of *C. albicans* cell wall was able to be changed by its environmental stress (Klis et al., 2014). The different mechanism and structure composition of *C. albicans* cell wall may be factors to its ability to resist the toxicity of nZVI particles. The specific study of nZVI effects on yeast such as *C. albicans* may be needed to explain the mechanism.

3.3 nZVI toxicity on microbes of aged nZVI

To better understand the toxicity effects of the residual nZVI, the colony count cytotoxicity assay was used on different types of microorganisms: *E. coli* and *P. aeruginosa* represented Gram negative bacteria, *S. aureus, B. Subtilis*, and *Rhodococcus* sp. represented Gram positive: and *C. albicans* represented yeast. Microorganisms with different nZVI treatments and exposure times were compared with the control group (not exposed to nZVI particles). The treatment results are shown in Figures 5-10.

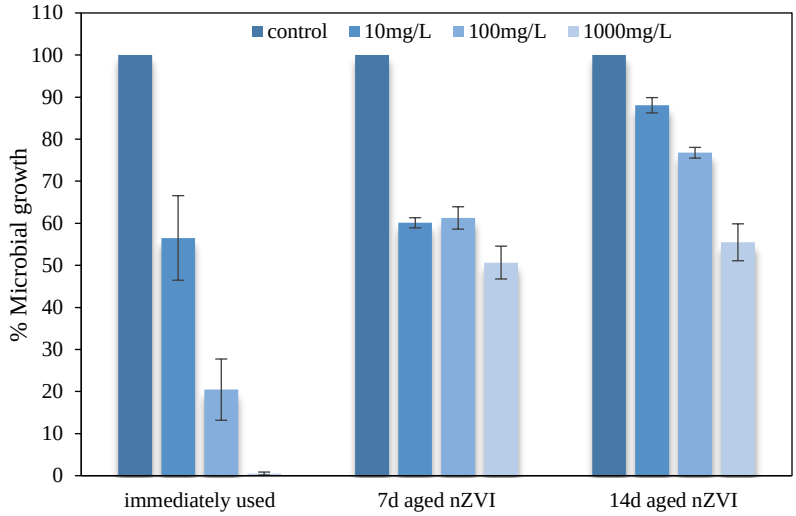

Figure 5. Percent *E. coli* growth in nZVI concentrations (10, 100, and 1,000 mg/L) at 0, 7, and 14 days aged nZVI.

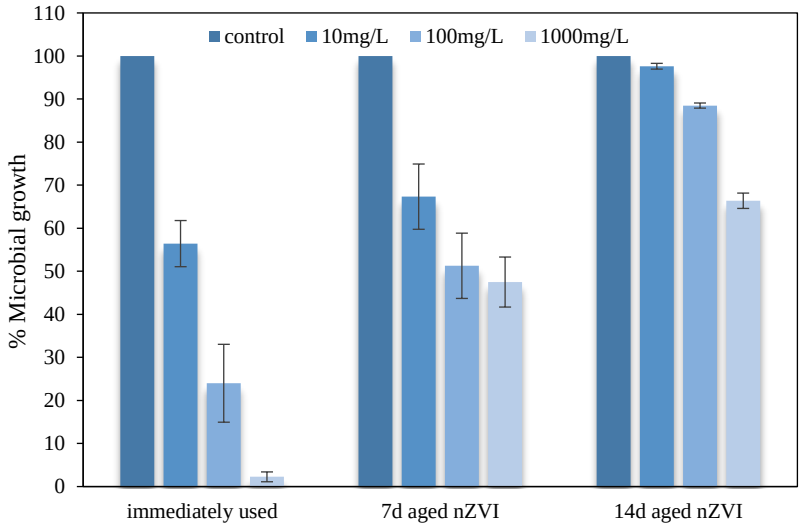

Figure 6. Percent *P. aeruginosa* growth in nZVI concentrations (10, 100, and 1,000 mg/L) at 0, 7, and 14 days aged nZVI.

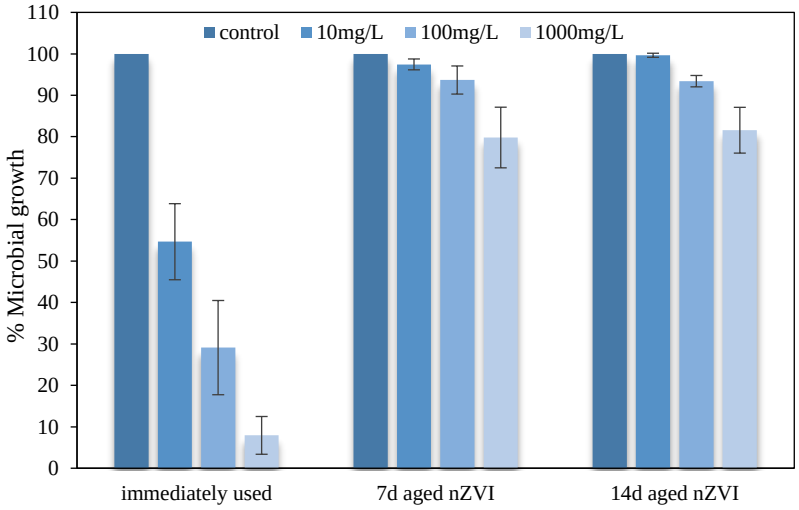

Figure 7. Percent *S. aureus* growth in nZVI concentrations (10, 100, and 1,000 mg/L) at 0, 7, and 14 days aged nZVI.

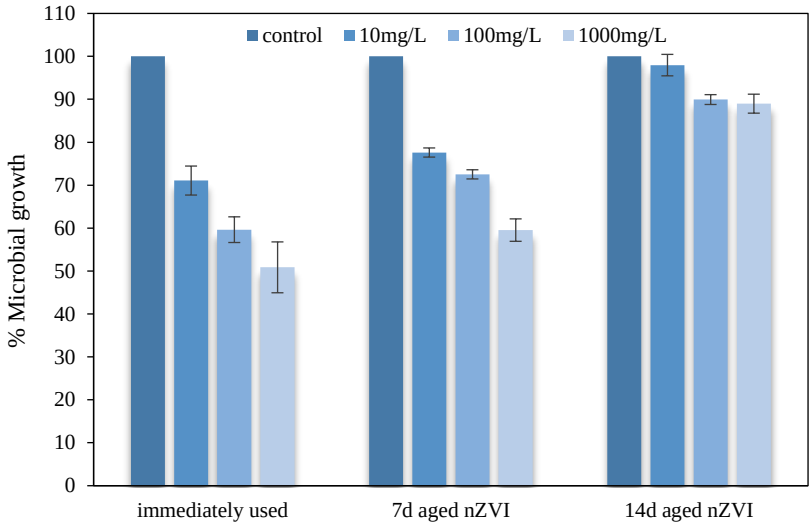

Figure 8. Percent *Rhodococcus* sp. growth in nZVI concentrations (10, 100, and 1,000 mg/L) at 0, 7, and 14 days aged nZVI.

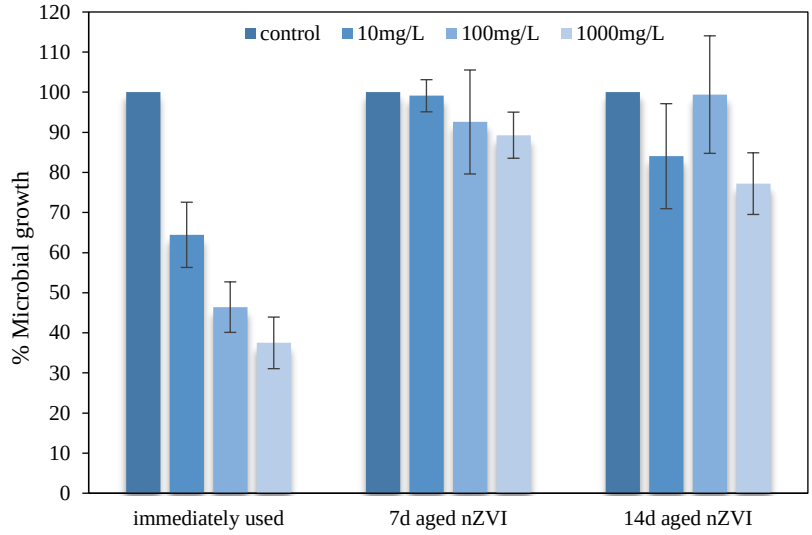

Figure 9. Percent *B. subtilis* growth in nZVI concentrations (10, 100, and 1,000 mg/L) at 0, 7, and 14 days aged nZVI.

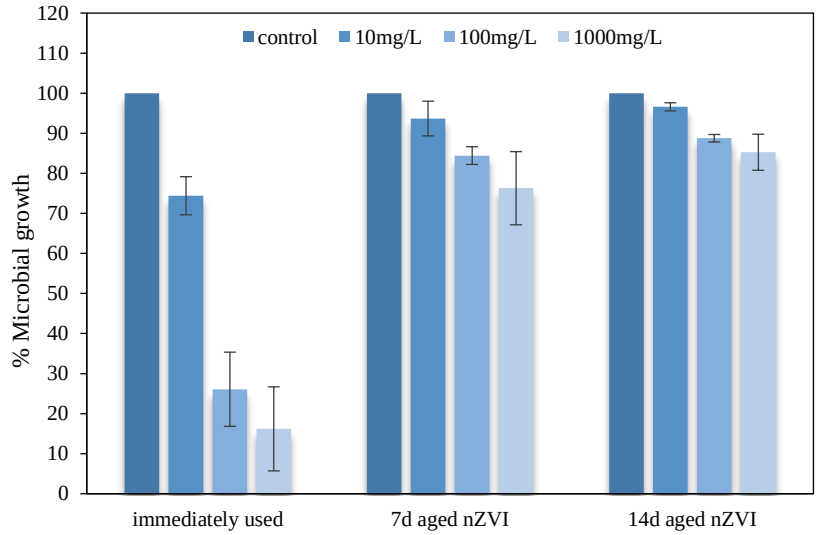

Figure 10. Percent *C. albicans* growth in nZVI concentrations (10, 100, and 1,000 mg/L) at 0, 7, and 14 days aged nZVI.

The bacteria were exposed to 7-day and 14-day old nZVI particles at different concentrations (10, 100, and 1,000 mg/L). As the results show, the toxicity was low in the nZVI long exposure groups. At 14-day aged nZVI, the results show that only minor effect was found even at the highest concentration (1,000 mg/L). Figure 11 presents the percentage of microbial growth with long-time exposure with 1,000 mg/L nZVI.

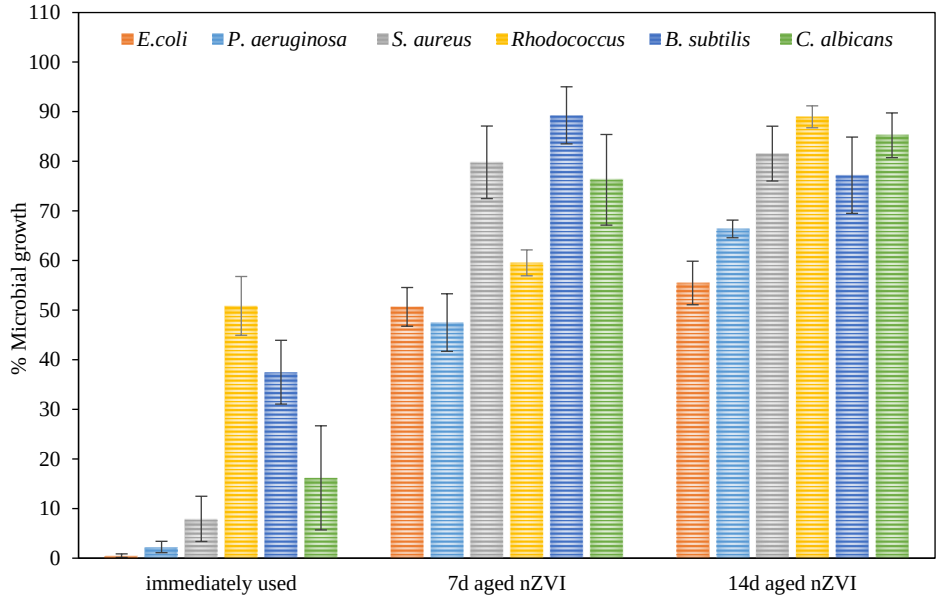

Figure 11. Microbial growth percent with long time exposure at 1,000 mg/L aged nZVI.

According to Figure 11, the percent growth of microorganism increased with aged nZVI particles exposing for 7 and 14 days, respectively. ANOVA statistical test for the three treatments showed significant differences at *p*-value less than 0.05 (*p*-value=0.00007). Therefore, the toxicity of nZVI particles to microbial growth was reduced after aging for long time. Therefore, the toxicity of aged nZVI particles had an inverse relation with microbial growth. All Gram positive bacteria were able to grow with 7-day aged nZVI and almost grew normally with 14-day aged nZVI. The reduction of toxicity of aged nZVI particles may be due to the change of the composition of particles. The outer shell of nZVI particles, which already reacted with surrounding environment, was changed to iron oxide, which has lower reactivity than fresh nZVI particles. The fresh particles shell consisted of Fe(0) that was ready to react (Li and Zhang, 2006; Yan et al., 2010). As seen in Figure 4, the reacted nZVI particle changed to iron oxide particles. Even though iron oxide particles were also able to produce ROS, their toxicity was far less than fresh nZVI particles (Diao and Yao, 2009).

Based on the results, the toxic effect of nZVI was dependent on the Gram type of bacteria as mentioned in the previous section. Moreover, the results also showed that the toxic effect of nZVI on microbial growth was only the instant effect. After the reactive power of the particles decreased, the toxic effect was much reduced. A significant decrease of aged nZVI toxicity to microorganism indicates that nZVI application to remediate contamination is not a long-term effect with small toxicity to microorganisms and nZVI particles will be naturally decomposed by the environment to iron oxide (iron rust), which is found normally in nature (Slunský, 2013).

However, there are many modifications of nZVI to increase its ability such as surface modification for increasing the particle mobility and bimetallic modification for increase the reactive power (Jiemvarangkul et al., 2011; O'Carroll et al., 2013; Bhuvaneshwari et al., 2017; Bensaida et al., 2021). These modified nZVI had higher reactivity and combined with other dangerous chemicals such as heavy metals. Moreover, residual wastes, that were created from used nZVI especially heavy metal adsorbed particles may be dumped into the environments with careless unsecured management. These modified nZVI and nZVI wastes can be expected to be the hazardous waste that is toxic to microorganism in natural environments. Therefore, studying the toxic effects of those particles and wastes to environmental microorganisms will be very important issues in the future.

There are many studies reporting the toxic effects of nZVI exposure in other microorganisms in the environment. A toxic effect of nZVI has been found in Pb-polluted soil treated by nZVI. After aged nZVI exposure in Pd-polluted soil, the ecotoxicological standardised test on *Caenorhabditis elegans* (*C. elegans*), a soil-dwelling bacterivorous nematode, indicated a decrease in the growth of *C. elegans*. However, a different event occurred in Zn-polluted soils treated by nZVI. There was no change in soil biodiversity and *C. elegans* were still able to growth after aged nZVI exposure. These specify that nZVI interaction with various types of pollutants should be considered to be a factor affecting microbial growth. The nZVI reaction significantly reducing *C. elegans* existence may be due to an increase in ROS concentration and the subsequent oxidative stress (Fajardo et al., 2015). nZVI inhibiting *C. elegans* growth was also found to be related to the particle concentration (Sacca et al., 2014). Reproductive toxicity assays presented that carboxymethyl cellulose (CMC)-stabilized nZVI (CMC-nZVI), nanoscale iron oxide (nFe_3O_4), and ferrous ion ($Fe(II)_{aq}$), a group of nZVI products, also significantly decreased the offspring of *C. elegans* due to the increase of reactive oxygen species (ROS) levels in its cells (Yang et al., 2016). These reports showed a similar trend with the results in this research and confirmed that the toxicity of nZVI was just an instant effect.

4. CONCLUSION

The experimental study presents that nZVI exposure to microorganisms affected the bacterial and yeast growth. The effect of nZVI on bacteria is related to type of microorganism and nZVI concentration. Gram-positive bacteria and yeast show more resistance to nZVI toxicity than Gram-negative bacteria. The toxicity effect of nZVI on bacteria is described by cytotoxicity mechanism: reactive oxidative species (ROS) production and oxidative stress. Additionally, the toxic effect of nZVI on microbial is a temporal effect. After a long period of exposure, the toxicity is significantly reduced. This research can confirm that nZVI particles, used in contamination treatment, have no long-term environment effect to microorganism. However, there still are several types of modified nZVI, such as surface modification, combined bimetallic particles and residual waste of nZVI, which need to be evaluated for their toxicity to microbes in the environment.

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