Impact of nanoscale zero valent iron on bacteria is growth phase dependent

Krittanut Chaithawiwata, b, Alisa Vangnai c, John M. McEvoy d, Birgit Pruess d, Sita Krajangpan e, Eakalak Khan f, * 

a International Postgraduate Programs in Environmental Management, Graduate School Chulalongkorn University, Bangkok 10330, Thailand 
b Environmental and Conservation Sciences, North Dakota State University, Fargo, ND 58108, USA 
c Department of Biochemistry, Chulalongkorn University, Bangkok 10330, Thailand 
d Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND 58108, USA 
e Medora Corporation, Dickinson, ND 58601, USA 
f Department of Civil and Environmental Engineering, North Dakota State University, Fargo, ND 58108, USA 

HIGHLIGHTS

• Log and decline phase Gram negative bacterial cells are more susceptible to nZVI.
• Susceptibility to nZVI is bacterial strain dependent.
• Higher concentrations of nZVI lead to increases in bacterial cell inactivation.
• Bacterial inactivation by nZVI is based on physical interaction.

ABSTRACT

The toxic effect of nanoscale zero valent iron (nZVI) particles on bacteria from different growth phases was studied. Four bacterial strains namely Escherichia coli strains JM109 and BW25113, and Pseudomonas putida strains KT2440 and F1 were experimented. The growth curves of these strains were determined. Bacterial cells were harvested based on the predetermined time points, and exposed to nZVI. Cell viability was determined by the plate count method. Bacterial cells in lag and stationary phases showed higher resistance to nZVI for all four bacterial strains, whereas cells in exponential and decline phases were less resistant to nZVI and were rapidly inactivated when exposed to nZVI. Bacterial inactivation increased with the concentration of nZVI. Furthermore, less than 14% bacterial inactivation was observed when bacterial cells were exposed to the filtrate of nZVI suspension suggesting that the physical interaction between nZVI and cell is necessary for bacterial inactivation.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Nanoscale zero valent iron (nZVI) particles are frequently used for environmental remediation due to their capacity to transform various toxic contaminants such as halogenated compounds and organo-pesticides (Grieger et al., 2010; Liu et al., 2005; Theron et al.,...
2. Materials and methods

2.1. Nanoscale zero valent iron particles

nZVI was purchased from Toda Co., Japan as reactive nanoscale iron particles. The obtained nZVI was synthesized by reduction of iron oxides using hydrogen gas. These particles have a zero-valent iron core and maghemite shell structure. nZVI, which was originally in a paste form, was dried and kept under nitrogen gas saturated condition to limit oxidation. The physical and chemical properties of nZVI that were examined included size, determined by transmission electron microscopy [JEOL, JEM-2100]; elemental composition, characterized by scanning electron microscopy-energy dispersive spectroscopy [JEOL, JSM-7600F]; and zeta potential in water, measured by a Malvern Zetasizer Nano ZS90.

2.2. Bacterial strains

Four bacterial strains were used in this study: Escherichia coli JM109 and E. coli K-12 strain BW25113 were from the Coli Genetic Stock Center at Yale University; Pseudomonas putida KT2440 (ATCC47054) and P. putida F1 (ATCC700007) were from the American Type Culture Collection. These species were selected because they are well characterized and, in the case of P. putida strains, are commonly found in environment (Dos Santos et al., 2004; Wu et al., 2011). The P. putida F1 strain also is a known TCE degrader (Wackett and Gibson, 1988). This study focused on Gram negative bacteria because Gram positives are relatively far less affected by nZVI (Diao and Yao, 2009; Chen et al., 2011). A single colony of E. coli grown on nutrient agar was inoculated into tryptic soy broth and incubated at 37 °C overnight with orbital shaking at 150 rpm (New Brunswick Incubator Shaker, Series 25). P. putida was cultured in a similar manner with the exception that the overnight culture was in brain heart infusion broth at 30 °C. Growth curves of the four bacterial strains were determined by adding 1% v/v of the overnight bacterial culture to 100 mL fresh medium and enumerating bacteria at different time points using the plate count method.

2.3. Bacterial inactivation by nZVI

Bacterial cells were collected by centrifugation (6000 × g for 5 min) at time points selected based on the growth characteristic of each strain (Table 1). The pellets were washed twice using 150 mM phosphate-buffered saline (pH 7.2), and finally resuspended in 50 mM carbonate buffer (pH 8.0). The final cell concentration was adjusted to 1 × 10^6–3 × 10^8 CFU mL^-1 in 25 mL of the same carbonate buffer followed by the addition of nZVI to a concentration of 1000 mg L^-1. The final cell concentration range is comparable to the values used in previous studies on impact of nZVI on bacterial cells and activities (Lee et al., 2008; Xu et al., 2010). The relatively high nZVI concentration was selected to represent a worst-case scenario and because the practical field concentration of nZVI is between 2 and 10 g L^-1, which is subsequently diluted once injected into contaminated sites. In addition, for JM109, the nZVI concentrations of 90, 200, 500, and 800 mg L^-1 were examined. Mixing was provided by a magnetic stirrer at 1500 rpm, and the number of viable cells was determined at 0, 5, 10, 15, 30, and 60 min by the plate count method. All experiments were carried out in triplicate. For each replicate, cells from different time points were collected from the same culture.

2.4. Inactivation using nZVI suspension filtrate

A filtrate of nZVI suspension was prepared by adding 1000 mg L^-1 of nZVI in 50 mM carbonate buffer (pH 8.0) followed by 1 h, 10 min, or 5 min of stirring using a magnetic stirrer at
1500 rpm, and filtration through a 0.22 μm pore size polycarbonate membrane filter (Isopore™, Millipore) to remove particles. Only JM109 cells were used in this experiment. They were collected and washed, as previously described, and resuspended in 25 mL of the filtrate to the concentration of $1 \times 10^6 - 3 \times 10^6$ CFU mL$^{-1}$. Cell viability was determined at 0, 5, 10, 15, 30, and 60 min by the plate count method. Cells from all four growth phases (all predetermined time points in Table 1) were tested. Experiments were carried out in triplicate. For each replicate, cells from different time points were collected from the same culture.

2.5. Statistical analysis and fifty percent lethal dose determination

Data from cell inactivation experiments were analyzed using the Statistical Analysis System (version 4.2). For each bacterial strain, the viability (survival) data for all time points of each growth phase (Table 1) were averaged to represent the results for each individual phase. The least significant difference t-test was used to compare the survival of cells from different growth phases. A significance level ($p$ value) of 0.05 was used as a criterion. Fifty percent lethal dose (LD50) for each growth phase of JM109 was determined based

---

**Fig. 1.** Inactivation of *E. coli* JM109 harvested from (a) lag (1 h) and exponential phases (2.5, 4, and 5.5 h), (b) stationary phase, and (c) decline phase using 1000 mg L$^{-1}$ of nZVI. Each data set in the graphs represents cell cultivation time (time point along the growth curve when the cells were harvested for use in nZVI exposure experiment).
3. Results and discussion

3.1. nZVI characteristics

Based on an examination of 350 random particles, the average size of nZVI was 28 nm with a size range of 10–70 nm. The percent size distribution based on number of particles was 16.9% (10–20 nm), 51.2% (20–30 nm), 21.1% (30–40 nm), 7.1% (40–50 nm), 3.1% (50–60 nm), and 0.6% (60–70 nm). The average zeta potential was –35 mV indicating moderate stability. The elemental composition by weight was 63.4% Fe, 32.4% O, 3.7% C, and 0.6% Al.

3.2. Effect of nZVI on bacterial cells from different growth phases

The survival of *E. coli* JM109 and BW25113 at different growth phases following treatment with nZVI is shown in Figs. 1 and 2. It should be noted that the natural growth and decay were not taken into account because the experiment was relatively short. In addition, the medium used in the experiment does not support
growth. The experiment with nZVI suspension filtrate resulted in minimal cell reduction indicating limited natural decay (See discussion in Section 3.4). Among different growth phases of JM109, early and mid-exponential (2.5 and 4 h cultivation) and decline phases (84 and 96 h cultivation) were most susceptible to nZVI with 3.5, 2.8, 2.8 and 4 log inactivation for cells harvested at 2.5, 4, 84, and 96 h respectively after 1 h exposure to nZVI (Fig. 1). However, cells in late exponential (5.5 h cultivation) showed lower inactivation, which is similar to cells in stationary phase. These results suggest that cells in the late exponential and stationary phases have greater resistance to nZVI, perhaps because of increased rpoS expression during late exponential and stationary phases, which regulates several genes in response to various stresses including oxidative stress (Yuste et al., 2006). The high resistance of bacteria in stationary phase might also result from cell adaptation to stressful environment since the phase normally has limited nutrient and necessary substrate for bacterial growth. Compared to exponential and decline phases, cells in the lag (1.7 log reduction) and stationary phases (2.4 log reduction) were more resistant to nZVI \( (p < 0.05) \).

Similarly, BW25113 was more resistant to nZVI in the lag and stationary phases compared to the exponential and decline phases (Fig. 2). Possible reasons for this observation are the same as those described above for JM109. Overall, BW25113 was more resistant to

![Fig. 3. Inactivation of *P. putida* KT2440 harvested from (a) lag (0.5 h) and exponential phases (2 and 3.5 h), (b) stationary phase, and (c) decline phase using 1000 mg L\(^{-1}\) of nZVI. Each data set in the graphs represents cell cultivation time (time point along the growth curve when the cells were harvested for use in nZVI exposure experiment).](image-url)
nZVI than JM109. After 30 min exposure to nZVI, all phases of BW25113 growth, with the exception of early log (1.1 log inactivation for 3.5 h cultivation time) and decline phases (1.4 log inactivation for 84 h cultivation time), showed less than 1 log inactivation; in contrast, JM109 inactivation was greater than 1 log for all phases.

Inactivation of _P. putida_ strains is shown in Figs. 3 and 4. Inactivation of strain KT2440 was less than 1 log for all treatments, which is considerably less than that of _E. coli_ strains. The F1 strain exhibited even greater resistance, with inactivation less than 0.4 log units. The overall resistance to nZVI of four bacterial strains ranged from high to low in the following order: _P. putida_ F1 > _P. putida_ KT2440 > _E. coli_ BW25113 > _E. coli_ JM109. These results suggest that the toxic effects of nZVI on bacterial cells are not only genus dependent, but also strain dependent. The limited effect of nZVI on _F1_ at all growth phases is optimistic since the strain is

---

_Fig. 4._ Inactivation of _P. putida_ F1 harvested from (a) lag (0.5 h) and exponential phases (2 and 3.5 h), (b) stationary phase, and (c) decline phase using 1000 mg L\(^{-1}\) of nZVI. Each data set in the graphs represents cell cultivation time (time point along the growth curve when the cells were harvested for use in nZVI exposure experiment).
known to degrade multiple organic contaminants including TCE.

3.3. Inactivation of bacterial cells by nZVI at different concentrations

The inactivation of JM109 was further investigated using lower nZVI concentrations, since this strain was least resistant to nZVI. Results indicated that the level of cell inactivation corresponded to the concentration of nZVI. Higher nZVI concentrations of 1,000, 800, and 500 mg L$^{-1}$ rapidly inactivated bacterial cell whereas 200 and 90 mg L$^{-1}$ of nZVI had limited effects on cell viability (Fig. 5, only data for 200 and 800 mg L$^{-1}$ of nZVI for stationary phase cells are shown as examples). Nonetheless, cell inactivation was observed at all nZVI concentrations examined in this experiment.

The LD50 of nZVI to JM109 cells in each growth phase based on 10 min incubation time was determined. Impact of nanoparticles on bacterial cells is a relatively new issue. Therefore, there is no typical or standard incubation time for LD50 determination. The 10 min incubation time was chosen because it provided a range of percent survival (corresponding to different nZVI doses) that can be used to determine LD50. The LD50 for cells in lag, exponential, stationary, and decline phases were 427, 380, 527, 329 mg L$^{-1}$, respectively. The high LD50 of stationary phase cells was consistent with the higher resistance of stationary phase cells to nZVI compared to other cells. This study demonstrates that the level of toxicity of nZVI to some bacterial strains is growth phase dependent. As a result, not choosing bacterial cells from suitable growth phase(s) when studying the impact of nZVI could provide inaccurate results. For example, as shown in this study, using bacterial cells from stationary phase for examining the impact of nZVI which is a common practice could lead to an underestimation of the toxicity. It is necessary to consider the growth phase that the cells are likely in when they are exposed to nZVI in actual cases before conducting the impact experiment.

3.4. Effect of nZVI suspension filtrate on bacterial survival

Inactivation using nZVI suspension filtrate was investigated in JM109 in order to determine the significance of physical interaction between bacterial cells and nZVI for cell inactivation. JM109 was selected because this strain showed the least resistance to nZVI. Very minimal cell inactivation (<14%) was detected following exposure of JM109 to nZVI filtrate for 1 h. Incubation of JM109 with the filtrate for 5 and 10 min caused negligible inactivation (data not shown).

4. Conclusions

Higher resistance to nZVI was observed in bacterial cells from lag and stationary phases. In contrast, cells from exponential and...
decline phases exhibited lower resistance to nZVI. The effect of nZVI on bacteria was genus and strain dependent, and nZVI concentration dependent. Physical interaction between cells and nZVI is necessary for cell inactivation. This study suggests that when studying impact of nZVI on bacterial cells, the growth phase of bacteria should be considered. Relying on one growth phase can result in over- or underestimation of the impact. To minimize impact of nZVI on bacterial degradation of contaminants, nZVI should be applied to established communities and avoided for sites that require bioaugmentation as supplemented cultures will have to undergo exponential growth, which is more susceptible to nZVI.

References


