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A Review of the Environmental Implications of *in situ* Remediation by Nanoscale Zero Valent Iron (nZVI): Behavior, Transport and Impacts on Microbial Communities

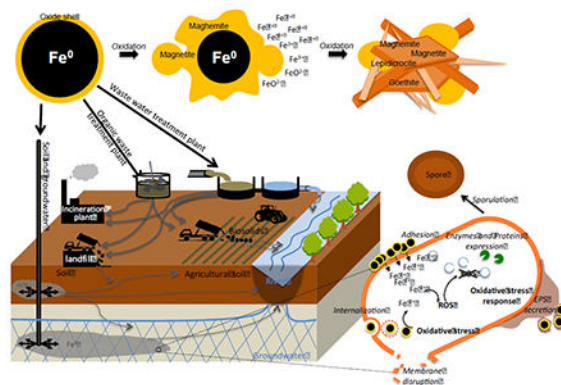
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Abstract

The increasing use of strategies incorporating nanoscale zero valent iron (nZVI) for soil and groundwater *in situ* remediation is raising some concerns regarding the potential adverse effects nZVI could have on indigenous microbial communities and ecosystem functioning. This review provides an overview of the current literature pertaining to the impacts of nZVI applications on microbial communities. Toxicity studies suggest that cell membrane disruption and oxidative stress through the generation of Fe^{2+} and reactive oxygen species by nZVI are the main mechanisms contributing to nZVI cytotoxicity. In addition, nZVI has been shown to substantially alter the taxonomic and functional composition of indigenous communities. However, because the physico-chemical conditions encountered *in situ* highly modulate nZVI toxicity, a better understanding of the environmental factors affecting nZVI toxicity and transport in the environment is of primary importance in evaluating the ecological consequences that could result from a more extensive use of nZVI.

Graphical abstract



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Keywords

Nanoscale zerovalent iron; Environmental impact; Toxicity mechanisms; Oxidative stress; Microbial community; nZVI transport; *In situ* remediation

1- Introduction

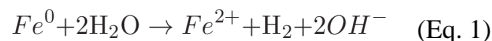
Nanoscale zero valent iron (nZVI) is the most commonly used nanomaterial in Europe and in the United States for soil and groundwater remediation. More recently, its use for wastewater remediation (Fu et al., 2014; Grieger et al., 2010; Mueller et al., 2012), and anaerobic digestion process enhancement (Carpenter et al., 2015; Hu et al., 2015) has also received growing attention. Due to its reduced size, nZVI has a higher reactivity towards a broad range of contaminants, including halogenated compounds, nitrate, phosphate, polycyclic aromatic hydrocarbons, and heavy metals (Fu et al., 2014; Mueller et al., 2012; Tosco et al., 2014; Wei-xian, 2003), and a higher mobility compared to its microscale counterpart. In addition, its application does not require excavation as highly concentrated nZVI slurries are directly injected underground, at or near the source of contamination. Consequently, nZVI is regarded as a promising remediation strategy suitable to a broad range of applications and environments. However, microorganisms, which are key players in many fundamental ecosystem processes, are the first exposed to nZVI particles. Therefore, potential issues related to long-term alteration of ecosystem functioning have to be considered and a thorough evaluation of the effect of nZVI on indigenous microorganisms is needed before further *in situ* deployment of nZVI remediation strategies. In the last decade, toxicity studies revealed that nZVI could exert some degree of toxicity towards microbial species and the effects of nZVI at the cellular and community levels are progressively being elucidated. In view of the abundant published literature on the topic, this review provides an updated overview of the potential impacts of the *in situ* deployment of nZVI on microbial communities (summarized in Figure 1). As characteristics and behavior of nZVI in the environment are essential for assessing its effects on microbial communities, a first section succinctly covers the main processes involved in nZVI chemistry. In a second section, toxicity studies conducted on microbial species are reviewed, and both the mechanisms likely mediating nZVI cytotoxicity, and the cellular defenses set off to counteract nZVI toxicity are presented. The following section covers the potential impacts of nZVI on the structure and ecological functions of microbial communities. Finally, based on current and potential future *in situ* applications of nZVI treatments, the transport and the possible routes of nZVI from injection points to non-target environments are considered.

2- Chemical behavior of nZVI *in situ*

2-1 Release of soluble iron and reactive oxygen species

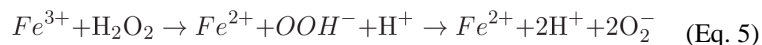
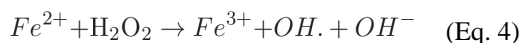
Even under highly controlled anaerobic conditions and despite the different protocols existing to synthesize nZVI, particle surface oxidation always occurs to some degree and results in the formation of a nanoparticle with a Fe⁰ core surrounded by an outer layer of iron oxide of at least 3 nm in thickness. Because kinetics of the initial stages of Fe⁰ oxidation are fast, once introduced into the environment the outer oxide layer of the

nanoparticle thickens relatively quickly (Crane and Scott, 2012). Ferrous (Fe^{2+}) and ferric (Fe^{3+}) irons, known to have cytotoxic effects, are initially released near the nanoparticle surface and progressively oxidize to form Fe(II) and Fe(III) oxides. This reaction evolves until the Fe^0 core is completely oxidized. The specific oxidation reactions of Fe^0 to soluble iron species are listed below for anaerobic (Eq. 1) and aerobic (Eq. 2 and 3) conditions.



Because the domain of stability for Fe^{3+} is narrower than for Fe^{2+} , under most environmental conditions, Fe^{2+} will be preferentially released upon nZVI injection. Fe^{2+} oxidation kinetics will vary greatly depending on environmental conditions and dictate the concentration of Fe^{2+} close to the injection point. The rate of Fe^{2+} oxidation can be influenced by various abiotic factors, including concentration in anionic species, dissolved oxygen, or other oxidants such as environmental pollutants. For instance, concentration in anionic species such as Cl^- in groundwater will reduce Fe^{2+} oxidation rate, resulting in a higher accumulation of Fe^{2+} (Adeleye et al., 2013).

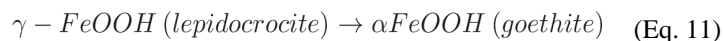
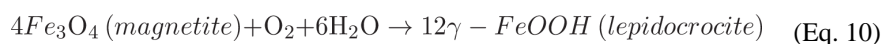
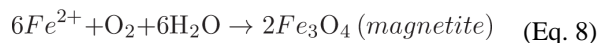
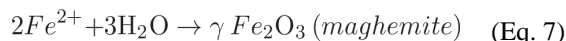
Fe^{2+} oxidation can also take place when hydrogen peroxide (H_2O_2) is present, such as inside a biological cell, resulting in the release of hydroxyl hydrogen radical (OH^\cdot) (Eq. 4), superoxide (O_2^-) (Eq. 5), or ferryl ion (FeO^{2+}) (Eq. 6; Ševc et al., 2011) through Fenton reaction. These highly reactive oxygen species (ROS) play a significant role in nZVI cytotoxicity.



2-2 Formation of insoluble iron oxide

Because Fe^{2+} and Fe^{3+} are only stable under specific environmental conditions, they are temporarily present in the environment, but ultimately oxidize to insoluble iron species such

as Fe₃O₄ (maghemite) (Eq. 7), Fe₃O₄ (magnetite) (Eq. 8), FeOOH (lepidocrocite) (Eq. 9 and 10), or goethite (Eq. 11).



High oxidation rates (e.g., oxic condition) will favor the formation of lepidocrocite (Eq. 9 and 10) or goethite (Eq. 11) (Kumar et al., 2014a). However, at lower oxidation rates (e.g., anoxic condition), soluble iron dehydroxylation will occur prior to oxidation, favoring the formation of crystalline green rusts, followed by magnetite as the end product of nZVI oxidation (Eq. 8) (Drever, 1988; Stumm and Morgan, 1996). The newly formed structures have been described as micronic aggregates of sphere, needle or board-shaped, composed of magnetite, lepidocrocite and goethite, respectively, with individual size between 20 nm and 1 μm (Greenlee et al., 2012; Su et al., 2013).

Kumar et al. (2014a), however, observed the formation of additional nanoparticles < 20 nm at the oxide layer surface of nZVI particles after 35 days under anaerobic conditions. Although never observed, detachment of oxide nanoparticles during the distortion of the oxide shell structure could potentially occur (Kumar et al., 2014a), and represent a potential hazard to microorganisms.

2-3 Effect of nZVI particle synthesis method and stabilization

Despite the numerous existing protocols, nZVI synthesis is mostly performed by chemical iron reduction. Synthesis parameters such as reduction rate, reagent concentrations (i.e., [Fe³⁺]/[BH₄]⁻ ratio) and concentration of nZVI nuclei (i.e., [Fe³⁺]), are known to greatly influence nZVI morphology, crystallinity, and specific surface area (i.e., size) (Hwang et al., 2011). Smaller nZVI particles usually result from synthesis under high reduction rate, and high reductant and nuclei concentration, while nZVI poor crystallinity is more associated to high nuclei concentration (Hwang et al., 2011). Other synthesis conditions including pH, temperature, the use of ethanol or additional cations have also been shown to influence nZVI

characteristics as well as its reactivity (Song et al, 2005). Therefore nZVI reactivity, hence toxicity, will likely depend on the synthesis methods and conditions used to produce the nanoparticles. In addition, because bare nZVI has low colloidal stability (Lowry and Casman, 2009; Tratnyek and Johnson, 2006), especially under commonly encountered environmental conditions (Saleh et al., 2008), nZVI particles synthesized for *in situ* remediation are typically coated with stabilizers. Polyacrylic acid, polyaspartate, chitosan, sodium oleate, and carboxymethyl cellulose coatings have been reported to significantly improve the colloidal stability of nZVI, reducing particle adhesion to mineral surfaces, and increasing nZVI mobility and overall reactivity (Phenrat et al., 2008; Saleh et al., 2007; Sirk et al., 2009). Although laboratory observations in columns of sand showed that the stabilizing effects of nZVI surface treatments could be maintained for an extended period of time compared to bare nZVI (Kim et al., 2009), biotic and abiotic degradation processes occurring *in situ* may progressively remove nanoparticle coating, modifying nZVI behavior and overall toxicity.

3- nZVI cytotoxicity

3-1 Toxicity studies on bacterial and fungal species

In the last decade, an increasing amount of studies evaluating the toxicity of nZVI on bacterial and fungal species have been conducted (Table 1). These studies primarily used *in vitro* toxicity assays to measure cell viability, cell growth, cell integrity, or biological activity of various microbial species exposed to nZVI, with exposure times ranging from 5 mins to 42 days, and nZVI concentrations ranging for 1 to 10,000 mg/l (Table 1). While a few studies reported no effect of nZVI, most of them found a strong to severe negative effect on cell viability, integrity and activity. Exposure to the highest nZVI concentrations tested did not lead to any toxic effects towards *Klebsiella planticola* or *Klebsiella oxytoca* (Fajardo et al., 2012; Saccà et al., 2013), but resulted in a severe toxic effect on *Bacillus nealsoni* (Fajardo et al., 2012). *Bacillus subtilis*, however, was found to be more resistant to nZVI toxicity than *Escherichia coli* (Chen et al., 2011) or *Pseudomonas fluorescens* (Diao and Yao, 2009), and *P. putida* displayed a lower sensitivity to nZVI than *E. coli* (Chaithawiwat et al., 2016). Within the same genus, differences in resistance were also reported. *B. cereus* displayed a lower resistance to nZVI toxicity compared to *B. nealsoni* (Fajardo et al., 2013, 2012), and while nZVI only had a transient toxic effect on *Pseudomonas stutzeri* (Saccà et al., 2014a), *P. fluorescens* exposed to lower doses was completely inactivated (Diao and Yao, 2009). Chaithawiwat et al. (2016) showed that different strains within the same species could also display differential sensitivity to nZVI. Additionally, they demonstrated that bacterial cells in stationary growth phase presented lower sensitivity to nZVI compare to cells harvested in lag or exponential phase, likely because *rpoS*, a transcriptional factor regulating various genes involved in cell stress response, was naturally more expressed during stationary growth (Battesti et al., 2011; Chaithawiwat et al., 2016; Kidarsa et al., 2013).

In comparison, fungal species seem to display a much higher tolerance to nZVI toxicity (Diao and Yao, 2009; Otero-González et al., 2013; Shah et al., 2010). Concentration up to 10,000 mg/l did not significantly affect the growth of *Aspergillus versicolor* (Diao and Yao, 2009), and although *Trametes versicolor* cellulolytic enzyme production, and

Saccharomyces cerevisiae metabolism decreased in the presence of nZVI, their viability was not significantly affected (Otero-González et al., 2013; Shah et al., 2010).

3-2 Toxicity mechanisms

Most of the literature suggests that cell membrane disruption and oxidative stress through the generation of Fe^{2+} and ROS by nZVI are likely the main mechanisms contributing to nZVI toxicity. In the majority of studies that used electron microscopy to evaluate damage to cell integrity, precipitation of nZVI or iron oxide on the cell wall or inside the bacterial cell was commonly observed (Table 1), suggesting that direct contact of nZVI with bacterial cell is required for nZVI to exert toxicity. Based on these observations, several mechanisms have been hypothesized. The high reducing power of nZVI may denature lipopolysaccharides, and electron and ionic membrane transport proteins, compromising the permeability of the membrane, and facilitating the entrance of toxic Fe^{2+} into the cell (Lee et al., 2008). Once internalized, Fe^{2+} could react with the H_2O_2 produced in mitochondria (i.e., through Fenton reaction), and form highly reactive oxygen species such as OH^\cdot , O^2 , or FeO^{2+} , leading to oxidative stress and subsequent cell death. Alternatively, nZVI could also complex with lipoteichoic acids, major constituents of the cell wall of Gram+ bacteria, or the anionic structures of the cell wall of certain bacterial species could stimulate the precipitation of iron oxides. Both these reactions could obstruct the porins of the cell outer membrane and prevent nutrient uptake (Chen et al., 2012; Diao and Yao, 2009). Although iron oxide accumulation was also observed on the cell wall of the fungus *Aspergillus versicolor*, its growth was not affected (Diao and Yao, 2009). This is likely due to the fact that fungi have an extremely rigid chitinous cell wall, acting as an extra shield against nZVI toxicity (Diao and Yao, 2009; Otero-González et al., 2013; Shah et al., 2010).

Another often-suggested mechanism contributing to nZVI toxicity involves the generation of highly reactive oxygen species, which accumulate in the cell environment and denature macromolecules including lipids, proteins, and nucleic acids, damaging intracellular structures and eventually leading to cell death. Oxidative stress could be directly caused by internalized nZVI, or indirectly through the release of toxic Fe^{2+} by nZVI adsorbed onto the cell, leading to a local increase in Fe^{2+} and penetration through the damaged membrane (Auffan et al., 2008), leading to oxidative stress and subsequent cell death as described above. Under aerated conditions, Fe^{2+} oxidizes more rapidly than it would under anaerobic conditions. Therefore, the contribution of Fe^{2+} to nZVI toxicity is higher under anaerobic conditions than under aerobic conditions (Kim et al., 2010). Significant inactivation of *E. coli*, mediated by Fe^{2+} was previously observed under de-aerated conditions (Lee et al., 2008), while no bactericidal effect was observed under aerated conditions.

Both mechanisms hypothesized for nZVI toxicity to bacteria imply a contact between ZVI nanoparticles and bacteria. Upon contact, redox reactions between iron and cell material may occur and/or reactive oxygen species that damage cells may be produced. The observed rate of inactivation will therefore be function of these intrinsic reaction rates and the rate of nZVI attachment to bacteria. If the intrinsic reaction in either case is assumed to be first order described by a rate constant, k_{int} , the overall rate constant for the reaction, k_{T} , can be described as sequential processes of transport, attachment and reaction (Barton et al., 2015).

Transport is described by a collision rate kernel, β , which describes nanoparticle transport to the vicinity of a bacterium. Attachment reflects the relative affinity α_{NB} of nanoparticles of concentration N for bacteria of concentration B. The observed apparent rate of the reduction reaction, k_T is related to the rates of heteroaggregation between nZVI and bacteria as:

$$k_T = \frac{k_{int} \alpha_{NB} \beta_B}{k_{int} + \alpha_{NB} \beta_B} \quad (\text{Eq. 12})$$

Surface treatments that increase mobility of nZVI may also decrease the affinity of nZVI for bacteria. Thus, it is possible that increasing nanoparticle mobility for better dispersal at contaminated sites will also decrease adverse effects on bacteria at these sites.

3-3 Cellular defenses against nZVI toxicity

As toxicity mechanisms of nZVI are being elucidated, resistance mechanisms or adaptative stress responses set off by bacterial cells to counteract nZVI toxicity are also being unraveled. *Bacillus* species respond to nZVI toxicity by forming spores (Fajardo et al., 2013, 2012). The appearance of a septum across *B. cereus* cells, which initiates the process of endospore formation, was observed after exposure to nZVI (Fajardo et al., 2013). This observation was supported by proteomic analyses that revealed a down regulation of flagellin, a structural protein of the flagellum, and phosphoglucosamine mutase, an enzyme catalyzing the synthesis of cell wall peptidoglycans and membrane lipopolysaccharides, likely preparing the cell to enter sporulation (Fajardo et al., 2013). The formation of spores in response to nZVI toxicity is likely a defense mechanism providing the cell with an extra protective barrier (i.e., hard coating surrounding the cell), preventing direct contact between nZVI and the bacteria. Another study by (Saccà et al., 2014a) showed that *B. stutzeri* exposed to nZVI down regulated the production of several porins and transporters proteins which play essential roles in nutrient and iron uptake through the cellular membrane, likely preventing iron to enter the cell and cause intracellular oxidative damages. *Klesbiella oxycota*'s high resistance to nZVI toxicity was attributed to the overproduction of tryptophanase, an enzyme converting tryptophan to indole, a signaling molecule produced under stressing environmental conditions and use to induce sporulation within the bacterial population (Saccà et al., 2013).

The production of extracellular polymeric substances [2] by certain bacterial species, such as *Agrobacterium* sp. and *Sphingomonas* sp. could also be one of the defense mechanisms developed by microbial species to mitigate nZVI toxic effects (Kim et al., 2012; Le et al., 2014). Excreted EPS adhere to nZVI and bacterial surface, limiting the contact between the two (Kim et al., 2012; Le et al., 2014). EPS are known to promote cell aggregation and adhesion in biofilms and flocs, protecting the cells against unfavorable environmental conditions. EPS also enhances communication between cells within the biofilm and floc formations, facilitating enzyme and metabolite secretion (Wingender et al., 1999).

Finally, oxidative stress response is the most often-cited defense mechanism triggered by nZVI toxicity. By using a strain of *E. coli* lacking superoxide dismutase, a superoxide-scavenging enzyme that transforms highly reactive O_2^- into O_2 or H_2O_2 , Auffan et al. (2008)

demonstrated that oxidative stress response was involved in *E. coli* resistance to nZVI. In the absence of superoxide dismutase, O_2^- would accumulate into the cell and denature macromolecules and intracellular structures. Catalase, an enzyme encoding by the *katB* gene, and also involved in cell ROS detoxification, catalyzes the transformation of H_2O_2 to H_2O and O_2 . This enzyme was overexpressed when *P. stutzeri* was exposed to nZVI (Saccà et al., 2014a, 2014b), suggesting that an oxidative stress response induced by H_2O_2 was involved in this case. In *B. cereus*, however, *katB* expression levels did not change after nZVI exposure, suggesting that other reactive oxygen species than H_2O_2 were involved nZVI toxicity, or that *B. cereus* quick entrance in sporulation prevented the cell to experience oxidative stress (Fajardo et al., 2013). Kim et al. (2010) also suggested that the primary ROS implicated in nZVI-induced oxidative stress in *E. coli* were OH. and FeO^{2+} rather than O_2^- and H_2O_2 . Proteomics analyses revealed that the defense mechanisms triggered by *P. stutzeri* involved the up-regulation of several other enzymes participating in oxidative stress response and iron homeostasis. For instance, the observed up regulation of delta-aminolevulinic acid dehydratase, an enzyme directly involved in cellular oxidative stress response, likely acted as an iron scavenger (Saccà et al., 2014a). Chaperonins and heat shock proteins, which are known to play a role when cells are exposed to various stressors, were also up regulated as well as several superoxide dismutases. In *B. cereus*, the up-regulation of dehydrogenases, implicated in ATP production, might have provided the additional energy required by the cell to enter in sporulation. In the same species, thioredoxin, a protein involved in oxidative stress response was also over produced (Fajardo et al., 2013). Transcriptomic and proteomic approaches are progressively shedding light on the complex enzymatic machinery involved in nZVI toxicity resistance and have proven extremely valuable in deciphering some of the mechanisms involved nZVI toxicity and cellular defenses.

3-4 Parameters influencing nZVI toxicity

3-4-1 nZVI dosage and exposure time—In general, toxic effects of nZVI towards bacterial species tend to increase with increasing nZVI concentrations (Auffan et al., 2008; Fajardo et al., 2012; Kim et al., 2012; Le et al., 2014). One study, however, showed that after 2 hours, 10,000 mg/l nZVI was less toxic to *B. cereus* than 1,000 mg/l, and that after 24 h, significant toxicity was still observed but only at the lowest concentration (Fajardo et al., 2013). nZVI aggregation is an important parameter to take into consideration when evaluating nZVI toxicity. nZVI aggregation rate will increase with increasing concentration, which will result in the formation of larger nanoparticle aggregates that ultimately sediment more rapidly from suspension (Lowry and Casman, 2009; Tratnyek and Johnson, 2006; Phenrat et al. 2007), resulting in reduced overall cytotoxicity.

Another important factor to consider in assessing nZVI toxicity is exposure time. For instance the toxicity of nZVI observed after 10 minutes on *P. stutzeri* faded after extended exposure times (Saccà et al., 2014a). Similarly, *Alcaligenes eutrophus* RNA content rapidly decreased during the first two days of incubation with nZVI. In the following days, however, growth gradually started again (An et al., 2010). In these particular studies, nZVI oxidation rather than sedimentation likely caused the decrease or loss of nZVI toxicity. Therefore, nZVI toxicity increases with increasing concentration but aggregation and oxidation are

likely to mitigate the toxicity effects of nZVI if excessive concentrations and longer exposure times are applied.

3-4-2 Effect of insoluble iron oxides—Upon injection into the environment, nZVI will ultimately oxidize. The speed and type of iron oxides formed mainly depend on the concentration of dissolved oxygen. Under aerobic conditions, the lifetime of nZVI (i.e., until nZVI reaches complete oxidation) can be as short as 2 hours, while under anaerobic conditions nZVI can take several months to completely oxidize (Li et al., 2010). The Fe⁰ content of nZVI gradually decreases with oxidation. *E. coli* exposed for 60 days to aged nZVI containing 20%, 7%, and 0% Fe⁰ (i.e., completely oxidized) displayed 5 log-, 2 log-inactivation, and no inactivation, respectively (Li et al., 2010), suggesting that nZVI toxicity decreases with decreasing nZVI Fe⁰ content, hence oxidation. Depending on the progression of nZVI oxidation, the proportion of the formed magnetite, maghemite and lepidocrocite varies. Exposure of *P. fluorescens*, *B. subtilis*, and the fungus *A. versicolor* to nZVI completely oxidized to FeOOH (lepidocrocite) did not result in any toxic effects even at concentrations up to 10,000 mg/l (Diao and Yao, 2009). Similar results were observed with *Agrobacterium* sp. (Zhou et al., 2014). At low concentrations (100 mg/l), magnetite was even found to promote denitrification by *Paracoccus* sp. by acting as a slow-release electron donor (Jiang et al., 2013; Liu et al., 2014). The toxicity of nZVI decreases with oxidation and ceases when the particle is completely oxidized because of the loss of electronic or ionic transfers at its surface (Auffan et al., 2009). Although never observed, it has been suggested that the potential detachment of oxide nanoparticles from aged nZVI surface could occur (Kumar et al., 2014a). While Auffan et al. (2008), and Otero-González et al. (2013) showed no toxic effect of maghemite nanoparticles on either *E. coli* or *S. cerevisiae*, in another study by Azam et al. (2012), the growth of *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *B. subtilis* was inhibited by maghemite nanoparticles at concentrations as low as 65 mg/l. Magnetite nanoparticles also presented some degree of toxicity towards *E. coli* at concentrations above 700 mg/l (Auffan et al., 2008). Therefore, even after nZVI passivation the secondary formation of iron oxide nanoparticles could also present some degree of toxicity towards microbial life.

3-4-3 Coating and presence of natural organic matter—Although a majority of toxicity studies have been conducted with bare-nZVI, nZVI particles used for *in situ* remediation are typically coated, and the type of coatings used to enhance nZVI particle colloidal stability has also been found to modulate nZVI toxicity. Chitosan and sodium oleate coatings displayed less toxicity towards *Alcaligenes eutrophus* than unmodified nZVI (An et al., 2010). Synthetic polymers such as polystyrene sulfonate, polyaspartate, or olefin maleic acid remarkably decreased nZVI toxicity towards *E. coli* and *Dehalococcoides* spp. (Li et al., 2010; Xiu et al., 2010a). A microcosm study even showed that polyaspartate coatings stimulated microbial growth in aquifer samples (Kirschling et al., 2010). Unlike bare-nZVI, these coated nanoparticles did not attach to the cells, suggesting that some coating stabilizers may limit the adhesion of nanoparticles to bacterial cells, likely by increasing electrostatic repulsions between the two (Li et al., 2010). However, although carboxy-methyl cellulose (CMC)-stabilized nZVI also had a reduced toxicity compared to bare-nZVI, it significantly adhered to *Agrobacterium* sp. cell surface but without causing

any damages to the membrane. A potential explanation for the observed reduced toxicity despite the physical contact between CMC-coated nanoparticles and bacterial cells, is that CMC might act as a hydroxyl radical scavenger, protecting the cell from oxidative stress (Zhou et al., 2014). Certain nZVI dispersants such as polyacrylic acid however, appeared to have a slight adverse effect on a TCE-degrading microbial community (Chang et al., 2014).

Natural organic matter (NOM) and humic acids, which are found in natural environments at high concentrations, have also been shown to attenuate nZVI toxicity. Chen et al., (2011) reported a mitigation of the bactericidal toxicity of nZVI towards *E. coli* and *B. subtilis* in the presence of humic acids as they adsorbed onto nZVI particles and bacterial cells, minimizing direct contact between the two. In agreement with these results, Li et al., (2010) showed that the presence of NOM reduced nZVI toxicity towards *E. coli*. Although nZVI with synthetic coating showed a higher reduced toxicity than NOM-nZVI, the same electric repulsion forces were involved in reducing nZVI cytotoxic effects. Therefore, the type of coating material and the presence of NOM are also factors to take into consideration when evaluating nZVI toxicity.

4- Effect on complex microbial communities

4-1 Impact of nZVI on community structure

Although valuable knowledge resulted from *in vitro* toxicity studies, given the complex microbial interactions existing in natural habitats and the high influence of environmental conditions, nZVI toxic effects are expected to be different *in situ* than *in vitro*. An increasing number of studies assessing the effect of nZVI on complex microbial communities from natural and engineered environments such as aquifer sediments, river water, soil, and activated sludge have been conducted (Table 2). These studies have integrated traditional microbial ecology and molecular techniques, often using a microcosm experimental setup, hence taking into account the interaction of nZVI with the microbial component and the environmental matrix.

In aquifer sediments, Kirschling et al., (2010) reported significant shifts in eubacterial diversity that were still apparent 250 days after nZVI addition. Kumar et al. (2014b) also reported a shift in the microbial community structure of aquifer sediments from a community dominated by *Acidithiobacillus ferrooxidans* and *Sulfobacillus*-related species to a community dominated by *Clostridium* and *Sporotalea propionica*-related species 130 days after nZVI addition. These results suggest that nZVI is likely to have long-term impacts on aquifer indigenous microbial community structure. In soil, nZVI was also reported to trigger drastic shifts in microbial community structure (Tilston et al., 2013). In a sandy clay loam, Fajardo et al. (2012) reported an increase in α -proteobacteria and Archaea and a decrease in β - and γ -proteobacteria abundances. In a different soil however, they noted an increase in β - and a decrease in ϵ -proteobacteria (Fajardo et al., 2015). Saccà et al. (2014b) who compared the effect of nZVI on the community structure of two different soils reported a decrease in Cytophaga-Flavo-bacteria and Firmicutes, and an increase in Actinobacteria in a loamy soil, whereas in a soil with higher sand content a decrease in α -proteobacteria and β -proteobacteria was observed. Using phospholipid fatty acid (PLFA) profiles, Pawlett et al. (2013) showed that the soil microbial biomass of Gram- bacteria and arbuscular mycorrhizal

fungi decreased as an effect of nZVI, and that shifts in community structure were more important in sandy soil than in clay. Therefore, soil texture and organic matter content seem to greatly influence the effect of nZVI on microbial communities.

In nitrifying activated sludge reactors amended with a sequential increase of nZVI from 0.2 to 20 mg/l over 56 days, pyrosequencing approaches revealed a slight shift in microbial communities exposed to nZVI (Ma et al., 2015). In activated sludge, shifts in microbial community composition increased with nZVI dosage from 20 to 200 mg/l (Wu et al., 2013), confirming the dose-dependent effect of nZVI. Once introduced into the system, nZVI reduces the oxidation-reduction potential (ORP), increases the pH of the environmental medium, and produces cathodic H₂ (Eq. 1), creating new environmental conditions that are more favorable for certain bacterial taxa than for others (Kischling et al., 2010; Kumar et al., 2014b). Therefore, in both a direct (i.e., toxicity effect) and indirect way (i.e., by inducing environmental physico-chemical changes), nZVI could select for certain bacterial species over others, hence inducing a shift in microbial community composition.

Microbial communities display critical functions involved in global nutrient cycling and because some bacterial species are functionally redundant, a shift in taxonomic composition does not necessarily translate in functional losses. Therefore, only looking at changes in community taxonomic composition gives an incomplete picture of the effect of nZVI on ecosystems. Some of the studies surveyed in the next section focus on specific functional communities of interest involved in nitrogen cycling, methanogenesis, sulfate reduction, and dehalogenation. Although most of these biological processes were studied in the context of wastewater treatment, results of these studies are also applicable to other natural ecosystems where these microbial processes occur.

4-2 Impact of nZVI on microbial functions

4-2-1 Nitrogen removal—Because nZVI generates a large quantity of H₂ under anaerobic conditions (Eq. 1), which could stimulate hydrogen-utilizing denitrifiers and hence accelerate the rate of biological denitrification, nZVI treatment used in combination with biotic denitrification is expected to improve denitrification rates in engineered water clean-up systems (Liu et al., 2014; Shin and Cha, 2008). A few studies assessing nZVI toxicity towards hydrogenotrophic denitrifiers were conducted and although a slight toxicity towards the hydrogenotrophic denitrifying bacterium *Parracoccus* sp. was observed at concentration close to 1,000 mg/l, the addition of 50 mg/l nZVI stimulated *Paracoccus* sp. denitrification rate and cell growth (Jiang et al., 2013; Liu et al., 2014). *Alcaligenes eutrophus*, which display a higher resistance to metallic ions, was much more resistant to nZVI toxicity (An et al., 2009; 2010), and presented an increased nitrate removal activity with increasing nZVI concentrations up to 700 mg/l (An et al., 2009). The primary goal of these studies though, was to develop a system combining nZVI-based nitrate removal and biological denitrification in order to increase biological denitrification and limit the amount of ammonium generated through the abiotic reduction of nitrate by nZVI. However, while some denitrifying bacterial cultures were positively affected by nZVI in terms of denitrification rates (Shin and Cha, 2008; An et al., 2009), most studies conducted on complex microbial communities showed that nZVI addition did not substantially affect the

overall denitrification process. Although the gradual addition of nZVI in a lab-scale nitrifying sequencing batch reactor resulted in a shift of the overall community structure, nitrate removal did not increase (Ma et al., 2015). In soil, Fajardo et al. (2012; 2015), who quantified the expression of functional denitrifying genes, *narG* and *nirS*, encoding nitrate and nitrite reductase, respectively, did not detect any effect of nZVI on nitrate removal. Similarly, in activated sludge, although the metabolic activity of the whole microbial community seemed to decrease with increasing doses of nZVI, no change in nitrate removal was detected (Wu et al., 2013).

Anaerobic ammonium oxidation (anammox) is another key biological process utilized for wastewater treatment. Under anaerobic conditions, anammox bacteria convert ammonium to nitrogen gas while using nitrite as an electron acceptor. Because of the obvious advantages of this process for water cleanup purposes, studies using nZVI in order to increase ammonium-removal yields were conducted. The widespread application of this process, however, is still hindered by a relatively long reactor startup time and the loss of a substantial fraction of the anammox bacterial biomass during reactor operation. Ren et al. (2015) showed that the addition of nZVI in an anammox upflow anaerobic sludge blanket reactor reduced startup time by 33.3%, and led to higher ammonium removal efficiencies. Indeed, nZVI, as a powerful reductant, consumed dissolved oxygen and generated ammonium *via* abiotic nitrate reduction, creating the favorable conditions for the faster growth of anammox bacteria. In addition, nZVI likely stimulated the secretion of EPS by anammox bacteria, promoting their aggregation in flocs and preventing their washout of the reactor (Ren et al., 2015).

4-2-2 Methanogenesis—nZVI has also been shown to increase methanogenesis and its use has been considered as a promising strategy to improve yield and quality of biogas production during anaerobic digestion. The fast release of H₂ by nZVI under anaerobic conditions favors the growth of hydrogenotrophic methanogens, which convert H₂ and CO₂ to CH₄. Thus, in addition to enhance CH₄ production, this process increases the quality and value of the biogas by fixing CO₂. Although substantial enhancement in CH₄ production in the range of 28 to 61% (Carpenter et al., 2015; Hu et al., 2015; Su et al., 2013) is usually achieved in the presence of nZVI, one study showed that high concentration of nZVI could also be detrimental to methanogens. Indeed, at higher nZVI concentrations, the faster release of hydrogen gas increases the hydrogen gas partial pressure at levels that favor hydrogenotrophic anaerobic processes, such as homoacetogenesis, over methanogenesis. In addition, the higher concentration of Fe²⁺ that might result from the addition of higher concentration of nZVI could inhibit methanogenesis (Yang et al., 2013).

4-2-3 Sulfate reduction—Biological sulfate-reduction is one of the most efficient ways to treat heavy metal-laden environments. In this approach, the production of sulfide, a by-product of biological sulfate reduction, leads to the sequestration of heavy metals through the formation of metal-sulfide precipitates. The use of nZVI in the context of heavy metal decontamination has been considered as an approach to enhance sulfate reduction, hence increase heavy metal sequestration. The decrease in dissolved oxygen and ORP, as well as the generation of H₂ that can be used as an electron donor by sulfate reducing bacteria

(SRB), is expected to create favorable conditions for the growth of SRB. In addition, abiotic heavy metal removal by nZVI could also occur through surface adsorption and subsequent precipitation. Kumar et al. (2014b) who investigated the effect of nZVI amendment on sulfate reduction, however, found that sulfate reduction was completely inhibited at nZVI concentrations above 500 mg/l. In agreement with these results, Barnes et al. (2010) found that doses above 300 mg/l led to a complete inhibition of sulfate reduction. On the contrary, Kirschling et al. (2010), who used quantitative PCR to measure SRB from aquifer material, reported an increase of the SRB population upon addition of 1,500 mg/l nZVI. The inconsistencies between these results are likely due to the syntrophic or competitive relationships existing between functional groups within the microbial community, as several functional groups compete for the cathodic H₂ generated by nZVI.

4-2-4 De-halogenation—*In situ* nZVI deployment may also affect indigenous microbial communities whose biodegradative potential could contribute to natural contaminant remediation. Therefore looking at the effects of nZVI on these particular communities is extremely relevant when assessing long-term impacts. As it does for biological denitrification, methanogenesis, and sulfate reduction, the cathodic H₂ generated by nZVI could serve as an electron donor for halorespirers, hence stimulating biological degradation of halogenated compounds. Laboratory-scale experiments, however, reported that TCE-degrading microbial activity was increasingly inhibited by increasing nZVI concentrations (Barnes et al., 2010; Xiu et al., 2010a, 2010b). nZVI coating, however, was shown to mitigate the negative effect of nZVI on degrading communities (Xiu et al., 2010a, 2010b). In line with these results, Tilston et al. (2013) showed that a chloroaromatic mineralizing population size and activity were reduced by the addition of nZVI, and even though population size recovered after 7 days, biological degradation remained inhibited. In a dioxin-contaminated soil, the addition of nZVI did not stimulate degradation, but promoted methanogenesis instead (Binh et al., 2015). Methanogens have shown to outcompete dechlorinators for the use of H₂ when higher H₂ concentrations and higher ORP are encountered. In this study, the dose of nZVI concentration used might have been too high, resulting in an excessive release of H₂ and fast increase of ORP which favored methanogens over dechlorinators (Binh et al., 2015; Xiu et al., 2010a, 2010b).

It should be noted, however, that the laboratory studies described above may not adequately reproduce the conditions encountered *in situ*. Indeed, a pilot-scale CMC-nZVI injection performed on a site contaminated with chlorinated organic compounds resulted in the successful degradation of chlorinated compound accompanied by a significant increase of *Dehalococcoides* spp. at the injection point and along the nZVI flow path (Kocur et al., 2015). This suggests that environmental variables such as soil texture and organic matter content, as well as nanoparticle coating, likely mitigate the negative effect of nZVI on *in situ* degrading-microbial communities.

5- Fate of nZVI in the environment

5-1 Transport

Understanding nZVI transport is essential to evaluate the potential of nZVI to travel from injection points to untargeted environmental compartments, and to assess the microbial community exposure to nZVI. While migration with relative ease from injection points may result in the dilution of nZVI, no transport will create hotspots of nZVI concentration close to the injection point. To date, most studies on nZVI migration have been performed in the context of groundwater and soil remediation, and have focused on the ability of nZVI to migrate within a contaminated plume. After injection, nZVI mobility is influenced by many interrelated environmental parameters, including ionic strength and composition, pH, O₂ concentration, presence of natural organic matter, and hydraulic conductivity of the environmental medium, as well as intrinsic properties of the nZVI particles such as surface coating, size, and concentration (Illés and Tombácz, 2006; Saleh et al., 2008; Gottschalk et al., 2010; Gottschalk and Nowack, 2011; Mueller et al., 2013; Mueller and Nowack, 2008). Although in groundwater systems, aggregation and adhesion to environmental particle surface are the primary factors limiting nZVI transport (Dong and Lo, 2013; Illés and Tombácz, 2006; Jung et al., 2014; Kocur et al., 2013; Petosa et al., 2010; Yin et al., 2012), high nZVI injection rates and high porosity of the aquifer material will enhance nZVI mobility (Kanel and Choi, 2007; Saleh et al., 2008). In porous media, nZVI can typically reach transport distances between 1 and 5 meters, which is significant in the context of groundwater remediation (Baalousha, 2009; Bennett et al., 2010; Busch et al., 2015; Kocur et al., 2014; Su et al., 2013; Johnson et al., 2013). Although at the scale of the groundwater ecosystem, this appears to be a limited distance, high concentrations of nZVI created at injection points can significantly change the local environmental conditions (e.g., ORP, pH, O₂ concentration), likely affecting both microbial community structure and functions within the nZVI reactive zone. In addition, dissolved species released by nZVI, such as Fe²⁺ will have the capacity to travel further (Shi et al., 2015), potentially expanding the zone of influence of nZVI. In comparison to groundwater systems, higher organic content, lower porosity and water flow usually prevailing in soil, limits nZVI transport capabilities, resulting in shorter nZVI travel distances (Dong and Lo, 2014).

5-2 Entrance points and potential routes to other environments

Pollutant remediation by nZVI is referenced in soils, groundwater, wastewater, and organic wastes (Fu et al., 2014; Mueller et al., 2012; Qu et al., 2013; Tosco et al., 2014; Tuomi et al., 2008). Although applications of nZVI beyond surface and subsurface remediation are extremely limited, organic waste stabilization, odor abatement, and biogas production are also referenced (Carpenter et al., 2015; Su et al., 2013). In this section all applications of nZVI are considered in order to understand nZVI life cycle, and evaluate its potential routes from entrance points to untargeted environments (Mitrano et al., 2015; Nowack, 2009; Som et al., 2010). However, because the detection of nanoparticles in complex environmental media has proven challenging (Hassellöv et al., 2008), data of transport of nZVI *in situ* are still lacking. Therefore, in this section, the routes taken by nZVI from application sites to other environmental compartments will be hypothesized according to general considerations.

In both groundwater and soil remediation applications, nZVI is deliberately and directly introduced at concentrations typically on the order of 10 g/l (Phenrat et al., 2009; Saleh et al., 2008), although concentrations as low as 0.75 – 1.5 g/l (Elliott and Zhang, 2001), and as high as 50 g/l (Tuomi et al., 2008) have been used. The groundwater remediation process consists of the direct injection of highly concentrated nZVI slurry through drilled boreholes in order to create subsurface reactive zones. In soil, however, treatments involve either subsurface injections or surface amendments. Detailed about field scale experiments can be found elsewhere (Bardos et al., 2015). Given the relatively low transport of nZVI in soil and groundwater, nZVI and oxide end products likely remain close to injection points. However, under particular environmental conditions, transfer of nZVI and oxidation products from groundwater or soil to adjacent surface waters could potentially occur. In this case, nZVI and end products will undergo dilution, aggregation, sedimentation, and further oxidation, likely resulting in the significant decrease of nZVI toxicity (Praetorius et al., 2012; Sun et al., 2014).

Compared to soil and groundwater, water and organic waste treatment plants, although the use of nZVI-based strategies in these systems is extremely limited in practice, can only be considered as a transient compartment for nZVI. Indeed, in organic waste treatment plants, nZVI and end products are expected to follow the treated waste disposal routes. However, the numerous uncertainties concerning the type of industrial plant, treated material, and treatment conditions make it difficult to assess the transfer of nZVI from these systems to other environmental compartments. In wastewater treatment plants, nZVI and end products could partition either to treated effluent or sludge. Given the high tendency of nZVI to adhere to other particles, nZVI is expected to accumulate, for the most part, in treated sludge, which can later be burnt in waste incineration plants, stored in landfills, or amended in agricultural soils as biosolids. The discharge of a small portion of nZVI in treated effluent cannot be excluded, as evidenced for TiO₂ nanoparticles (Kiser et al., 2009). Soluble iron ions, however, are expected to be released in the treated effluent and ultimately in surface water. In organic waste treatment plants, nZVI and end products routes into the environment can also be assumed to follow organic wastes disposal routes (i.e., similar to wastewater treated sludge).

Finally, the behaviour of nZVI in landfill is not well documented. To date, almost no data exist on the fate and state of nanoparticles in waste incineration processes, and leaching from landfills. From landfill, the transfer to soil and surface water of Ag, TiO₂, Zn, CNT and fullerene nanoparticles has been modelled, and predicted to be negligible for the most part (Sun et al., 2014). Depending on the landfill types (e.g., inert materials, stable materials, or reactive materials), nZVI and end products could undergo cement consolidation, acid washing, grinding and could also be in contact with various organic and inorganic materials (Mueller et al., 2013). Based on these considerations, the stabilization and/or destruction (dissolution) of nZVI in landfill is most likely, and the same behaviour is expected in waste incineration plant.

6- Conclusion and perspectives

The implication of nZVI-based strategies for environmental applications must be thoroughly evaluated before the technology can be further deployed *in situ*. Because microorganisms are ubiquitous and essential for the functioning of ecosystems, studying the impact of nZVI on microbial communities is of primary importance in evaluating the ecological consequences that could result from the more extensive use of nZVI. Toxicity studies showed that nZVI could exhibit strong to severe toxic effects towards certain microbial species, and although nZVI toxicity is likely to be mitigated by the physico-chemical conditions encountered *in situ*, substantial shifts in taxonomic composition were reported. From a functional perspective, the introduction of nZVI into the environment will likely disturb the equilibrium between the functional microbial groups carrying out important biogeochemical processes. In addition, based on current and potential future *in situ* applications of nZVI treatments, untargeted ecosystems might be affected by nZVI. Based on most of the studies conducted to date, whether the focus is directed towards the mechanisms involved in nZVI toxicity at the cellular level, or towards the impact of nZVI at the microbial community level, the environmental conditions encountered *in situ* definitely have an important role in how nZVI affects microbial communities and ecosystem functions. Therefore, future studies should consider bringing greater emphasis on the effect of environmentally relevant factors on nZVI toxicity in order to provide a better understanding of the ecological impacts related to the use of nZVI.

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Highlights

- nZVI toxicity towards microbes is highly modulated by environmental conditions.
- Cell membrane disruption and oxidative stress contribute to nZVI toxicity.
- Microbes have developed adaptative response to nZVI toxicity.
- nZVI disturb microbial community taxonomic and functional composition.

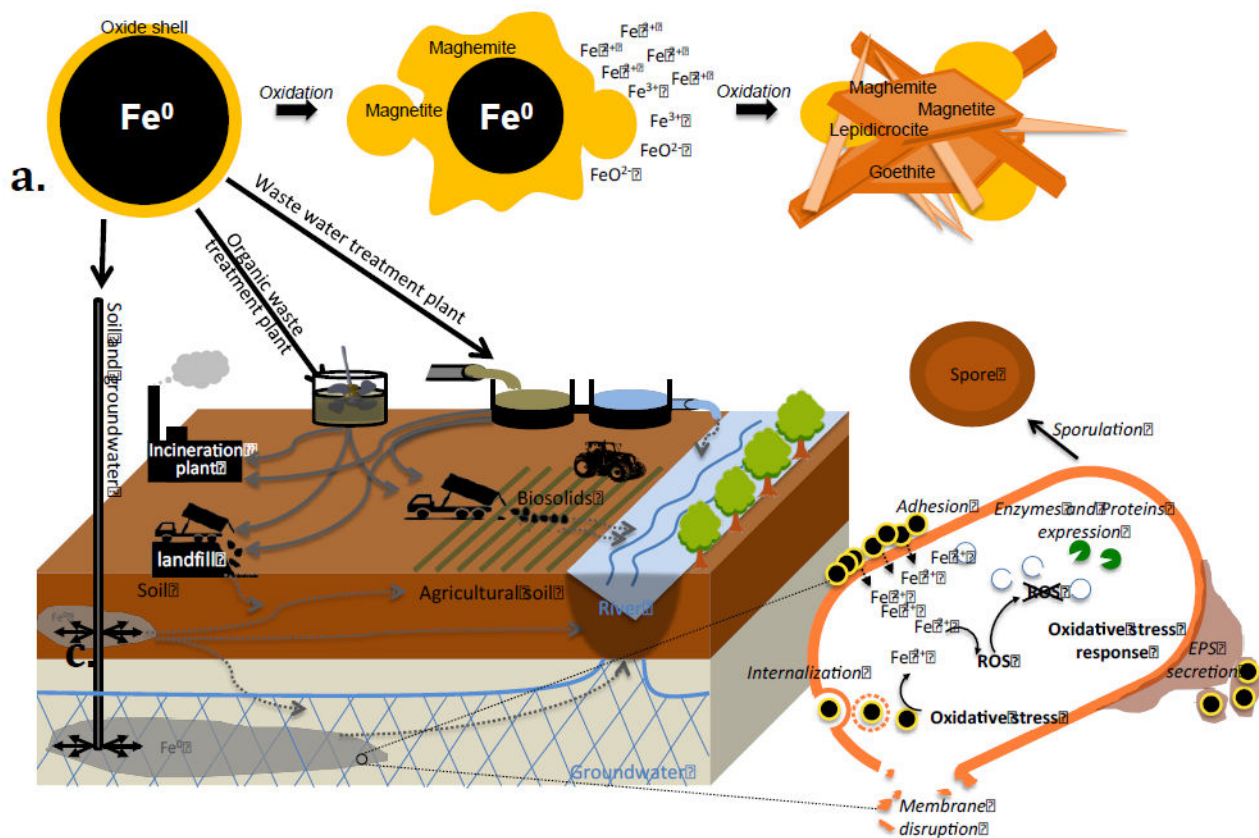


Figure 1. nZVI oxidation, cytotoxicity, cellular defense mechanisms mediated by nZVI, and potential routes of nZVI in the natural environment

Illustration of (a.) nZVI oxidation process, (b.) bacterial toxicity and defense mechanisms, and (c.) potential routes followed by nZVI in the environment. Plain grey arrows represent deliberate injection or amendment of nZVI, and deliberate transport of material potentially containing nZVI. Dashed grey arrows represent potential non-deliberate transport of nZVI in non-target environments. Abbreviations: EPS, Extracellular polymeric substances; ROS, Reactive oxygen species.

Table 1

Summary of *in vitro* toxicity studies conducted on bacterial and fungal species.

Species tested	nZVI characteristics	nZVI concentration	Experimental conditions	Measured biological parameters	Methods	Effect	Reference
<i>Klebsiella planticola</i> (Gram -)	nZVI (NANOFER 25S *) < 50 nm	1,000-10,000 mg/l	0.2-24 h-exposure, no shaking, aerobic	Cell viability Biological activity	CFU assay O ₂ consumption	No effect on viability and activity. Attachment of nZVI to cell surface.	Fajardo et al., 2012
<i>Klebsiella oxytoca</i> (Gram -)	nZVI (NANOFER 25S *) 80-120 nm	1,000-10,000 mg/l	0.2-24 h-exposure, no shaking, aerobic	Cell integrity Cell viability Cell integrity	TEM/SEM CFU / Liquid-to-plate	No significant cell damage. No bactericidal and negligible bacteriostatic effects.	Sacca et al., 2013
<i>Pseudomonas fluorescens</i> (Gram -)	Home-made bare-nZVI 20-30 nm	100-10,000 mg/l	5 min-exposure, vigorous shaking, aerobic/anaerobic	Cell viability Cell integrity	CFU assay SEM	Complete inactivation at all concentration tested. Iron precipitate coating on cell surface.	Diao and Yao, 2009
<i>Pseudomonas putida</i> (Gram -)	nZVI (Toda Kogyo Corp., Japan) 10-70 nm	1,000 mg/l	5-60 min-exposure, mixing, aerobic	Cell viability	CFU assay	More severe toxicity on exponential and decline growth phases.	Chaithawiwat et al., 2016
<i>Pseudomonas stutzeri</i> (Gram -)	nZVI (NANOFER 25S *) < 50 nm	1,000-10,000 mg/l	0.2-48 h-exposure, aerobic	Cell viability	CFU assay	Toxic effect is strain-dependent. Slight toxicity only after 0.2 h-exposure.	Sacca et al., 2014a
<i>Alcaligenes</i> (Gram -)	Home-made bare-nZVI, chitosan-nZVI, sodium-oleate-nZVI ~80 nm	650 mg/l	1-9 d-exposure, shaking, aerobic	Gene expression level (<i>pykA</i> , <i>gyrA</i> , <i>katB</i>) Protein transcription level	RT-qPCR 2D SDS-PAGE	No effect on activity. Increase of oxidative stress response.	An et al., 2010
<i>Agrobacterium sp.</i> (Gram -)	Home-made bare-nZVI, CMC-nZVI	100-250 mg/l	1-6 h-exposure, shaking, aerobic	Protein transcription level Cell integrity Biological activity	2D SDS-PAGE TEM Total RNA measurement	Attachment on nZVI on cell surface. Down-regulation of membrane transport proteins. Up-regulation of oxidative stress response proteins.	Zhou et al., 2014

Species tested	nZVI characteristics	nZVI concentration	Experimental conditions	Measured biological parameters	Methods	Effect	Reference
<i>Agrobacterium</i> sp. PH-08	80-120 nm			Cell integrity	TEM	Drastic damage of cell membrane.	Le et al., 2014
	nZVI (NaBond, China)	100-10,000 mg/l	1-6 h-exposure, shaking, aerobic	Cell viability	CFU assay	11.4 and 32% decrease in cell viability after 6 h-exposure to 100 and 1,000 mg/l nZVI, respectively.	
<i>Escherichia coli</i> (Gram -)	36.3 ± 2.3 nm			Gene expression level (Catechol dioxygenase)	RT-qPCR	Only slight effect on degradation capabilities.	Chaithawiwat et al., 2016
	nZVI (Toda Kogyo Corp., Japan)	1,000 mg/l	5-60 min-exposure, mixing, aerobic	Cell integrity	Propidium Iodine TEM/SEM	Attachment of nZVI cell surface without disruption or internalization.	
	10-70 nm			Cell viability	CFU assay	More severe toxicity on exponential and decline growth phases.	
<i>Escherichia coli</i>	Home-made bare-nZVI	7-700 mg/l	1 h-exposure, aerobic	Cell viability	CFU assay	Toxic effect is strain-dependent.	Auffan et al., 2008
<i>Escherichia coli</i>	320 ± 30 nm			Cell viability	CFU assay	Severe toxicity (~75% inactivation at 70 mg/l nZVI).	Li et al., 2010
	Bare nZVI (Toda kogyo Corp., Japan)	1-2,000 mg/l	5-60 min-exposure, shaking, aerobic/anaerobic	Cell viability	CFU assay	1.8 and 5.2-log inactivation for 7 and 28% Fe ⁰ nZVI, respectively. <0.2-log inactivation with coated nZVI after 1h-exposure.	
<i>Escherichia coli</i>	PSS-nZVI			Cell viability	TEM	Attachment on nZVI on cell surface.	Lee et al., 2008
	PA-nZVI			Cell viability	CFU assay	Severe toxicity under de-aerated.	
	NOM-nZVI			Cell integrity	TEM	Slight toxicity under aerated conditions.	
<i>Escherichia coli</i>	Home-made bare nZVI	1.2-110 mg/l	2-60 min-exposure, mixing, aerobic/anaerobic	Cell viability	CFU assay	Significant cell damage.	Chen et al. 2011
	10-80 nm			Cell viability	CFU assay	Severe toxicity (60%, 70%, ~100% inactivation after 1, 2, and 4 h-exposure, respectively).	
<i>Escherichia coli</i>	nZVI (Toda kogyo Corp., Japan)	1,000 mg/l	1-4 h-exposure, shaking, aerobic	Cell viability	CFU assay	80% inactivation.	Chen et al., 2012
<i>Escherichia coli</i>	50 nm-5 µm			Cell viability	CFU assay		Kim et al., 2010
	Home-made bare-nZVI, electrospayed nZVI	5,000 mg/l	5 min-exposure, vortexing, aerobic	Cell viability	CFU assay	Serious damage of cell membrane and respiratory activity under de-aerated conditions, but not under air-saturated conditions.	
<i>Escherichia coli</i>	Home-made bare-nZVI	1.2-110 mg/l	1 h-exposure, mixing, aerobic/anaerobic	Cell viability	Live/Dead viability		Y.-M. Kim et al., 2012
10-80 nm				Cell viability	CFU assay	Grow normally up to 2,500 mg/l nZVI.	
<i>Sphingomonas</i> sp. PH-07 (Gram -)	Home-made bare - nZVI	500-10,000 mg/l	4 d-exposure, shaking, aerobic	Cell viability	CFU assay		

Species tested	nZVI characteristics	nZVI concentration	Experimental conditions	Measured biological parameters	Methods	Effect	Reference
<i>Bacillus nealsonii</i> (Gram +)	SSA: $33.2 \pm 1.2 \text{ m}^2 \cdot \text{g}^{-1}$					Does not grow > 5,000 mg/l.	
	nZVI (NANOFER 25S *)	1,000-10,000 mg/l	0,2-24 h-exposure, no shaking, aerobic	Cell viability	CFU assay	Slight and high toxicity at 5,000 and 10,000 mg/l respectively.	Fajardo et al., 2012
	< 50 nm			Cell integrity	O ₂ consumption	Reduced metabolic activity.	
<i>Bacillus subtilis</i>	nZVI (Toda kogyo Corp., Japan)	1,000 mg/l	1-4 h-exposure, shaking, aerobic	Cell viability	TEM/SEM	Cell content leakage.	Chen et al., 2011
	50 nm-5 μm				CFU assay	Strong toxicity (20%, 70%, ~100% inactivation after, 1, 2, and 4 h-exposure, respectively).	
<i>Bacillus subtilis</i>	Home-made bare-nZVI	100-10,000 mg/l	5 min-exposure, vigorous shaking, aerobic	Cell viability	CFU assay	Strong bactericidal effect (100, 95, and 80% inactivation at 100, 1,000, and 10,000 mg/l nZVI, respectively).	Diao and Yao, 2009
<i>Bacillus cereus</i> (Gram +)	20-30 nm			Cell integrity	SEM	Massive needle-shaped coating on cell surface.	
	nZVI (NANOFER 25S *)	1,000-10,000 mg/l	0,2-24 h-exposure, no shaking, aerobic	Cell viability	CFU assay	Negative effect on cell viability, but no effect after 48 h-exposure.	Fajardo et al.,
	< 50 nm			Protein transcription level	2-D2D SDS-PAGE	Up-regulation of oxidative stress response proteins. Down-regulation of cell wall and motility proteins.	
<i>Paracoccus</i> sp. YF1 (Gram -)	Home-made bare-nZVI	50-1,000 mg/l	20 h-exposure, shaking, aerobic	Gene expression level (<i>pykA</i> , <i>gyrA</i> , <i>katB</i> , <i>nis</i> , <i>narG</i>)	RT-qPCR	No change in genes expression level.	Jiang et al., 2015
				Cell integrity	TEM	Early sporulation.	
				Nitrate removal	UV-spectrophotometry	50 mg.l ⁻¹ nZVI promoted and >50 mg/l decreased growth and nitrate removal.	
<i>Paracoccus</i> sp. YF1	Home-made bare-nZVI	1,000 mg/l	20 h-exposure, shaking,	Cell growth	Spectrometry (OD _{600nm})	Attachment of nZVI to cell surface.	
	20-80 nm			Cell integrity	SEM		
				Nitrate removal	UV-spectrophotometry	Decrease of cell growth and nitrate removal.	Jiang et al., 2013
<i>Paracoccus</i> sp. YF1	Home-made bare nZVI	50-1,000 mg/l	24 h-exposure, shaking, aerobic/anaerobic	Cell growth	Spectrometry (OD _{600nm})	Attachment of nZVI to cell surface.	
	20-80 nm			Cell integrity	SEM		
				Nitrate removal	UV-spectrophotometry	Initial retardation in cell growth and nitrate removal.	Liu et al., 2014
	20-80 nm			Cell growth	Spectrometry (OD _{600nm})	Attachment of nZVI to cell surface.	
				Cell integrity	SEM		

Species tested	nZVI characteristics	nZVI concentration	Experimental conditions	Measured biological parameters	Methods	Effect	Reference
<i>Saccharomyces cerevisiae</i> (Ascomycetous yeast)	nZVI (Skyspring Nanomaterials Inc., USA) 40-60 nm	100-1,000 mg/l	20 h-exposure, shaking, aerobic	Biological activity Cell integrity	O ₂ consumption	Low overall toxicity.	Otero-Gonzales et al., 2013
<i>Aspergillus versicolor</i> (Ascomycetous)	Home-made bare-nZVI 20-30 nm	100-10,000 mg/l	5 min-exposure, vigorous shaking, aerobic	Cell viability Cell integrity	Toxicity yeast assay/Flow cytometry CFU assay SEM	Negligible cell damage. No toxic effect. Yellow-brown coating observed on cell surface.	Diao and Yao, 2009
<i>Trametes versicolor</i> (Basidiomycetous Fungi)	nZVI (Sun innovation, Inc., USA) 25 nm	0.1 mM Fe ⁰	42 d-exposure, shaking, aerobic	Cell growth Cell activity	Dry weight Lignocellulolytic and Cellulolytic enzyme production	No effect on growth. Decrease of cellulolytic enzymes production.	Shah et al., 2010

Abbreviations: CFU, colony forming unit; CMC, carboxyl methyl cellulose; NOM, natural organic matter; PA, poly aspartate; PSS, polystyrene sulfonate; RT-qPCR, reverse transcription-quantitative PCR; SEM, scanning electron microscopy; SSA, surface specific area; TEM, transmission electron microscopy; MALDI-TOF, matrix-assisted laser desorption ionization tandem time-of-flight; SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *GirA*, *katB*, *narG*, *nirS*, and *pykA* genes encode for gyrase (DNA replication enzyme), catalase (cellular detoxification enzyme), nitrate reductase, nitrite reductase, and pyruvate kinase (glycolysis enzyme), respectively.

* NANOFER 25S: nZVI coated with polyethylene glycol sorbitant monostearate with a percentage Fe⁰ of 14-18% (NANOIRON s.r.o., Czech Republic).

Table 2

Summary of studies conducted on the effect of nZVI on microbial communities.

Community studied	nZVI characteristics	nZVI concentration	Experimental conditions	Measured biological parameters	Methods	Effect	Reference
Agricultural land	nZVI (NANOFER 25S [*])	34 g Fe ⁰ /kg	72 h-exposure, shaking, aerobic.	Community composition	FISH	Significant shifts in community composition.	Fajardo et al., 2012
	< 50 nm			Gene quantification and expression level (<i>nirS</i> , <i>narG</i> , <i>gyrA</i>)	RT-qPCR	No effect on denitrifying populations and gene expression level.	
Brownfield soil	nZVI (NANOFER 25S [*])	100 g/kg	13 d-exposure.	Community composition	FISH	Increase in β- and decrease in α-proteobacteria.	Fajardo et al., 2015
				Gene expression level (<i>nirS</i> , <i>narG</i> , <i>KatB</i> , <i>pykA</i>)	RT-qPCR	Overexpression of <i>katB</i> , and underexpression of <i>pykA</i> . No change in <i>narG</i> and <i>nirS</i> expression.	
Arable land sandy, loam, and clay soil	Home-made CMC-nZVI	0.27 g Fe ⁰ /kg	4 month-exposure, 15°C, dark.	Community composition	PLFA profiles	Community composition shifts higher in sandy soil. Shift in community functionality only in clay soil.	Pawlett et al., 2013
	10 ± 5.2 nm			Community functional profile	MSIR	Reduction of Gram – bacteria and AMF.	
				Microbial biomass	Fumigation-extraction		
Arable land soil	PAA-nZVI (Golder Associates Inc., USA)	10 g/kg	28 d-exposure, 25°C, dark	Community composition	DGGE	Shifts in Community composition.	Tilston et al., 2013
	12.5 ±0.3 nm			Chloroaromatic mineralizing microbial population activity	¹⁴ C-based MPN technique (¹⁴ C-UL-2,4-D substrate)	Chloroaromatic biodegradation activity reduced.	
Loamy-sand, and loam soil	nZVI (NANOFER 25S [*])	17 g/kg	7 d-exposure, 21 ±0.5°C, dark.	Community composition	FISH	No major change in community composition.	Sacca et al., 2014b

Community studied	nZVI characteristics	nZVI concentration	Experimental conditions	Measured biological parameters	Methods	Effect	Reference
Aquifer sediment	PAA-nZVI, bare-nZVI (Toda Kogyo Corp., Japan)	1,500 mg/l	250 d-exposure, shaking, 23 ±2°C, dark.	Gene expression level (<i>katB</i> , and <i>pykA</i>) Community composition	RT-qPCR DGGE	Overexpression of <i>katB</i> and <i>pykA</i> . Significant shift in microbial community composition.	Kirschling et al., 2010
Aquifer sediment	27.5 nm nZVI (Toda Kogyo Corp., Japan)	500-3,000 mg/l	130 d-exposure, 16±2°C, dark, anaerobic	Microbial population dynamics (bacterial and archaeal 16S rDNA, <i>dsrA</i> gene) Community composition	qPCR 16S environmental cloning-sequencing	Increase of SRB and methanogens. Shifts in microbial community.	Kumar et al., 2014
Activated sludge	Home-made bare-nZVI 70-100 nm SSA: 20.71 m ² ·g ⁻¹	20-200 mg/l	9 h-exposure, SBR, anaerobic/aerobic	Community composition	Pyrosequencing	No drastic changes in community composition. ROS increased with increasing nZVI doses.	Wu et al., 2013
Activated sludge	nZVI (NANOFER 255 *) 46 ± 10 nm	0.1 20 mg/l	56 d-exposure, SBR, 20°C	nZVI cytotoxicity Microbial activity Sludge surface integrity	Intracellular ROS and LDH ATP cellular content	No effect of nZVI on LDH release. ATP content decreased with increasing nZVI doses. Slight damage of sludge surface at 200 mg/l.	Ma et al., 2015

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Abbreviations: AMF, arbuscular mycorrhizal Fungi; CMC, carboxyl methyl cellulose; DGGE, denaturing gradient gel electrophoresis; EDS, energy dispersive X-ray spectroscopy; FISH, fluorescent *in situ* hybridization; LDH, lactate dehydrogenase; MSIR, multiple substrate-induced respiration; PAA, polyacrylic acid; PLFA, phospholipid fatty acid; ROS, reactive oxygen species; RT-qPCR, reverse transcription-quantitative PCR; SBR, sequencing batch reactor; SEM, scanning electron microscopy; SRB, sulfate-reducing bacteria. *GirA*, *katB*, *narG*, *nirS*, and *pykA* genes encode for gyrase (DNA replication enzyme), catalase (cellular detoxification enzyme), nitrate reductase, and pyruvate kinase (glycolysis enzyme), respectively.

* NANOFER 255: nZVI coated with polyethylene glycol sorbitan monostearate with a percentage Fe⁰ of 14-18% (NANOIRON s.r.o., Czech Republic).