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Residual impact of aged nZVI on heavy metal-polluted soils

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HIGHLIGHTS

- No negative effects on soil properties were observed after exposure with aged nZVI
- The nZVI treatment produced an increase in soil Fe availability
- Pb-nZVI soil showed changes in biodiversity, enhanced oxidative stress and Pb toxicity
- Increased biological activity and decreased Zn toxicity were observed in Zn-nZVI soil
- Impact of aged nZVI exposure mainly depends on the heavy metal contaminant

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ABSTRACT

In the present study, the residual toxicity and impact of aged nZVI after a leaching experiment on heavy metal (Pb, Zn) polluted soils was evaluated. No negative effects on physico-chemical soil properties were observed after aged nZVI exposure. The application of nZVI to soil produced a significant increase in Fe availability. The impact on soil biodiversity was assessed by fluorescence *in situ* hybridization (FISH). A significant effect of nZVI application on microbial structure has been recorded in the Pb-polluted soil nZVI-treated. Soil bacteria molecular response, evaluated by RT-qPCR using exposure biomarkers (pykA, katB) showed a decrease in the cellular activity (pykA) due to enhanced intracellular oxidative stress (katB). Moreover, ecotoxicological standardised test on *Caenorhabditis elegans* (*C. elegans*) showed a decrease in the growth endpoint in the Pb-polluted soil, and particularly in the nZVI-treated. A different pattern has been observed in Zn-polluted soils: no changes in soil biodiversity, an increase in biological activity and a significant decrease of Zn toxicity on *C. elegans* growth were observed after aged nZVI exposure. The results reported indicated that the pollutant and its nZVI interaction should be considered to design soil nanoremediation strategies to immobilise heavy metals.

1. Introduction

Heavy metal contamination of soil is an important concern because of its toxicity, which threatens human life and the environment. Anthropogenic contamination of soils by heavy metals (e.g., Pb and Zn) occurs from many sources, such as mining, atmospheric deposition and the application of sludge, mineral fertilisers and pesticides (Khan et al., 2010; Tosco et al., 2014). Thus, the main objective of many *in situ* remediation strategies is to reduce the mobile fraction of metals and metalloids in the soil that could reach the groundwater or be taken up by soil organisms. In this sense, several strategies have been used to promote the immobilisation of metals in soil (Kumpiene et al., 2008). Among these strategies, nano-sized zero-valent iron particles (nZVI) are considered to be an effective option for the treatment of contaminated soil and groundwater systems (Mueller et al., 2012), mainly targeting chlorinated organic contaminants (El-Temshah et al., 2013), inorganic anions, metals (Fajardo et al., 2012; Gil-Díaz et al., 2014a; Mueller et al., 2012), and metalloids (Gil-Díaz et al., 2014b). Although the benefits of this strategy are evident, governments must weigh the associated environmental risks because currently available ecotoxicology data are not conclusive (Handy et al., 2012).

Widely used standardised ecotoxicity testing methods have been applied to assess the effects of nZVI on standard test organisms (Handy et al., 2012). Among these, *Caenorhabditis elegans* (*C. elegans*), a soil-dwelling bacterivorous nematode, has been used as a test eukaryote organism for investigating not only metal and nanoparticle toxicity (Shen et al., 2009; Wu et al., 2012) but also complex matrices such as soil (Höss et al., 2012; Saccà et al., 2014b). Nevertheless, to better understand the toxicological effects of nanomaterials on environmental biota, soil health and functionality, it is necessary to define new relevant

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endpoints in addition to these classical tests and traditional chemical analyses. The assessment of soil microbial biodiversity, a parameter that is directly related to soil functionality and health, has effectively been performed by quantifying the main bacterial phylogenetic groups present in soil using fluorescence *in situ* hybridisation (FISH) (Fajardo et al., 2012; Saccà et al., 2014b).

In addition, the changes in the expression of relevant genes/proteins under nanomaterial exposure have provided key insights into the molecular mechanisms of action of engineered nanoparticles (Fajardo et al., 2012; Saccà et al., 2013; Saccà et al., 2014a). The study of several of these exposure biomarkers, which are of particular relevance to cellular activity (*narG*, *nirS*, *pykA*, *katB*), and their transcriptional changes under nanomaterial exposure might provide a comprehensive understanding of the impact of nanotechnology on microbial functionality. The *pykA* gene encodes pyruvate kinase, the key enzyme in glycolysis that catalyses the final step of this metabolic pathway. Pyruvate kinase controls the consumption of metabolic carbon for biosynthesis and the utilisation of pyruvate for energy production; the regulatory properties of this enzyme are of extreme importance for living organisms. Catalase (encoded by the *katB* gene) is a key component in the cellular detoxification of reactive oxygen species (ROS)-induced oxidative stress. ROS are generated naturally in all aerobic organisms via the respiratory chain and via exposure to redox-active chemicals, such as nanoparticles (i.e., nZVI) (Ševcu et al., 2011). *narG* and *nirS* are genes encoding the denitrification enzymes nitrate reductase and cytochrome cd1-containing nitrite reductase, which are involved in the nitrogen cycle, a key geochemical process in the soil ecosystem.

Previous column experiments have proved the effectiveness of nZVI for the *in situ* immobilisation of heavy metals, which reduces their potential leachability, as a strategy to prevent their transport into deeper soil layers, rivers, and groundwater (Gil-Díaz et al., 2014c). However, regarding nZVI-induced soil toxicity scarce data have been reported, providing preliminary information on the effects of nZVI on soil biota, but most of the reported studies have been conducted under *in vitro* conditions and/or considering short-term exposure. The impact of nZVI treatment on soil properties and functionality remains unclear, even more so regarding long-term exposure to nZVI. Usually, nZVI reactivity, degradation efficiency and likely toxicity are reduced after ageing due to the oxidation process that takes place upon contact with soil, but the extent and duration of the potential negative effects of this treatment should be addressed (El-Temshah et al., 2013).

In this work, the residual impact of aged nZVI, used as remediation strategy for metal immobilisation (Pb, Zn), has been assessed in soil after a leaching experiment simulating two years of annual rainfall. Changes in the soil physico-chemical and biological characteristics, gene expression profiles and biodiversity have been evaluated as relevant endpoints; moreover, standardised tests on *C. elegans* have been conducted to evaluate the ecotoxicological effects of the strategy.

2. Materials and methods

2.1. Commercial zero-valent iron nanoparticles

Iron nanoparticles (NANOFER 25S) were commercially synthesised and supplied by NANO IRON s.r.o. (Rajhrad, Czech Republic) as an aqueous dispersion of stabilised nZVI (coated with sodium polyacrylic acid 3%), with an average particle size of <50 nm. The Fe(0) content was 14–18%, and 2–6% iron oxides when produced. Additional details about the physical and chemical characteristics are available at www.nanoiron.cz. Nanoparticles were used immediately after receipt.

2.2. Soil microcosm setup

The soil used in the present study came from a previous column experiment that was performed to evaluate the immobilisation and leaching of Pb and Zn in soils treated with nZVI (Gil-Díaz et al., 2014c). Briefly, the soil was artificially contaminated with Pb or Zn at 192 and 250 mg/kg, respectively (higher levels than maximum allowed concentrations by the Spanish legislation RD1310/1990 for soils with pH < 7). Spiked soils were consolidated for 40 days. The column experiment was conducted, including columns with unpolluted soil, columns with polluted soil (Pb or Zn), and columns with polluted soil treated with nZVI at 10% (called Pb-nZVI or Zn-nZVI). Three independent columns were used per condition. After 72-h of interaction soil-nZVI, de-ionised water was run through the soil periodically by gravity flow for 10 days up to 1120 mL, which supposes two years of typical annual rainfall from central Spain. After this period, the soil was removed from the column, homogenised, and divided into two samples: one for the biological analysis and the other one was air dried for the physico-chemical determinations. The physico-chemical soil properties (pH, conductivity, organic matter, porosity, water holding capacity, and available macronutrients, Ca, Mg, Na and K) were analysed according to the official Spanish methodology for soil analysis (MAPA, 1994). The total Fe, Pb and Zn levels in the soil samples were determined by AAS after acid digestion in a microwave reaction system (Multiwave 3000, Anton Paar GmbH, Graz, Austria), as previously described by Gil-Díaz et al. (2014c). Iron content in the most available fractions (e.g., exchangeable and linked to carbonates) was analysed in the soils after applying a sequential extraction procedure (Gil-Díaz et al., 2014c). Soil respiration was determined using the m-Trac 4200 system (SY-LAB, Pukersdorf, Austria). Statistical treatment of the data was performed using SPSS release 19.0.0.1 (SPSS Inc., IBM Company). The means were compared through one-way ANOVA using Tukey's test ($p < 0.05$).

2.3. Soil microbial biodiversity

The phylogenetic composition of the soil microbial communities was analysed in the soil samples by FISH using published probes and protocols (Fajardo et al., 2012; Saccà et al., 2014b). Further details on the probes used are available at proBase (Loy et al., 2003). The final results are expressed as the percentage of DAPI (4,6-diamidino-2-phenylindole)-positive cells that hybridised with the fluorescent probes.

All data are expressed as the mean ± standard error of the mean. The results from the FISH assays were analysed statistically using Student's *t*-test (at $p < 0.01$, $n = 3$) and GraphPad Prism 5 software (San Diego, CA, USA).

2.4. Expression analysis of biomarker genes by quantitative PCR (qPCR)

The expression of the biomarker genes was analysed using four sets of primers synthesised and developed by PrimerDesign, Ltd. (Southampton, UK) to specifically amplify the *narG*, *nirS*, *pykA* and *katB* sequences of strains *of Bacillus cereus* and *Pseudomonas stutzeri*. Primer sequences and RT-qPCR conditions are described in Fajardo et al. (2013) and Saccà et al. (2014a). Briefly, total RNA was extracted from the reference strains using the UltraClean Microbial RNA Isolation Kit (MO Biomedicals, LLC.) and from the incubated 0.5 g soil samples using the FastRNAProSoil-Direct Kit (MO Biomedicals, LLC.). Specific reverse transcription (RT) for each target mRNA was performed according to the iScriptTM Select cDNA Synthesis Kit manufacturer's instructions using the specific reverse primers. The levels of target gene expression were reported relative to the levels of an internal 16S rDNA reference gene. Standard curves for each of the target genes were included in each assay. Ratios above or below 3-fold were considered significant.

2.5. Toxicity assays (C. elegans)

The *C. elegans* wild type strain N2 was obtained from the *Caenorhabditis* Genetic Centre (University of Minnesota, St. Paul, MN, USA). *C. elegans* were maintained on nematode growth medium

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