Ecotoxicology and Environmental Safety 123 (2016) 81–88

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Phytotoxicity of ionic, micro- and nano-sized iron in three plant species

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ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form
17 May 2015
Accepted 21 July 2015
Available online 29 July 2015

Keywords:
Lepidium
Sinapis
Sorghum
Nano-zerovalent iron
Nanoecotoxicology
Phytotoxicity

ABSTRACT

Potential environmental impacts of engineered nanoparticles (ENPs) can be understood taking into consideration phytotoxicity. We reported on the effects of ionic ($FeCl_3$), micro- and nano-sized zerovalent iron (nZVI) about the development of three macrophytes: *Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum*. Four toxicity indicators (seed germination, seedling elongation, germination index and biomass) were assessed following exposure to each iron concentration interval: 1.29–1570 mg/L ($FeCl_3$), 1.71–10.78 mg/L (micro-sized iron) and 4.81–33,560 mg/L (nano-iron). Exposure effects were also observed by optical and transmission electron microscopy. Results showed that no significant phytotoxicity effects could be detected for both micro- and nano-sized zerovalent irons, including field nanoremediation concentrations. Biostimulation effects such as an increased seedling length and biomass production were detected at the highest exposure concentrations. Ionic iron showed slight toxicity effects only at 1570 mg/L and, therefore, no median effect concentrations were determined. By microscopy, ENPs were not found in palisade cells or xylem. Apparently, aggregates of nZVI were found inside *S. alba* and *S. saccharatum*, although false positives during sample preparation cannot be excluded. Macroscopically, black spots and coatings were detected on roots of all species especially at the most concentrated treatments.

1. Introduction

Engineered nanomaterials (ENMs) are highly preferred for a broad spectrum of applications due to their unique properties. Engineered nanoparticles (ENPs) have a promising use in many areas including catalysis, optics, biology, agriculture, and microelectronics (Wu et al., 2012; Libralato et al., 2013; Corsi et al., 2014; Libralato, 2014; Minetto et al., 2014). Further applications are currently focused on environmental remediation due to their likely performance in contamination removal and toxicity mitigation (Gavaskar et al., 2005; Tratnyek and Johnson, 2006). Iron-based ENPs stimulated research for engineering applications especially for treating polluted water and groundwater (Tang and Lo, 2013) including both inorganics and organics (Crane and Scott, 2014; Mar Gil-Díaz et al., 2014; Zeino et al., 2014). Their impact is still highly empirical and limited to nanoparticles' elemental composition, size and stability showing both positive and negative effects. The high reactivity of iron-based ENPs, and in particular of nano-zerovalent iron (nZVI), in association with their high specific surface area made them suitable to immobilise and degrade contaminants in soils (Chang et al., 2007; Machado et al., 2013). Thus, the use of nZVI for soil clean-up purposes could pose potential hazards for macrophytes and soil organisms (Ma X. et al., 2010; Ma Y. et al. 2010). In all ecosystems, plants are the basic component playing a crucial role in the fate and transport of ENPs in the environment through plant uptake and bioaccumulation (Monica and Cremonini, 2009).

Although in remediation activities, terrestrial macrophytes could be directly exposed and potentially affected by nZVI, effect data are still scarce despite the current use of this technique (Li et al., 2015). Zhu et al. (2008) found that *Cucurbita maxima* grown in an aqueous medium absorbed, translocated and accumulated Fe_3O_4 ENPs, but this event did not occur with *Phaseolus limensis*

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http://dx.doi.org/10.1016/j.ecoenv.2015.07.024
0147-6513/

under the same testing conditions. This suggested that the biological effect could be species-dependent. Lee et al. (2010) showed that Fe_3O_4 ENPs in *Arabidopsis thaliana* did not significantly affect seed germination and the number of produced leaves, while the root elongation was negatively influenced at all exposure concentrations (400, 2000, and 4000 mg Fe_3O_4/L). Kim et al. (2014, 2015) investigated the effect of nZVI on *A. thaliana* root elongation showing an enhanced growth by 150–200% at 0.5 g/L compared to the blank. Further studies on *A. thaliana* evidenced that nZVI triggered high plasma membrane H^+-ATPase activity resulting in a 5-fold higher stomatal opening than in unexposed plants (Kim et al., 2015). Mushtaq (2011) observed that concentrations of Fe_3O_4 ENPs within 100–5000 mg/L were able to significantly reduce *Cucumis sativus* root development compared to controls suggesting the presence of stressing conditions. Phytotoxic effects of Fe_3O_4 ENPs were assessed in lettuce (*Lactuca sativa*), radish (*Raphanus sativus*) and cucumber (*C. sativus*) (Wu et al., 2012) evidencing median effective concentrations (EC50) of more than 5000 mg/L for lettuce and radish, and of 1682 mg/L for cucumber, respectively. For all species, the germination index was significantly different from standard conditions showing seedling inhibition effects. Seeds of *Linum usitatissimum*, *Lolium perenne* and *Hordeum vulgare* were used to investigate the potential inhibition effects of nZVI (El-Temsah and Joner, 2012). Concentration of 2 and 5 g/L of nZVI completely inhibited seed germination, while no detrimental effects on plants were observed at concentrations <250 mg/L. Pereira et al. (2013) observed changes in root and shoot lengths, number of lateral roots, photosynthetic pigments, and internal CO_2 concentration in four rice cultivars when nano-iron exposure in the growth medium increased from 4 to 9 mM. Similarly, other authors found differences in plant growth, nutrient uptake, and lateral roots morphology in *Ipomoea pescaprae* and *Canavalia rosea* when exposed to bulk $FeSO_4$ (Siqueira-Silva et al., 2012). Ma et al. (2013) showed that concentrations higher than 200 mg/L of nZVI reduced plant growth and biomass in *Typha latifolia* and hybrid poplar (*Populous deltoids* × *Populous nigra*). Trujillo-Reyes et al. (2014) investigated the effects of Fe_3O_4 ENPs in *L. sativa*. No physiological change was detected compared to negative controls. Iron ions or ENPs (10 and 20 mg/L) had low or no negative effect on cell membrane integrity and chlorophyll content. Mukherjee et al. (2014) studied ZnO iron doped (Fe@ZnO) ENPs toxicity in *Pisum sativum* (L.) analysing seed germination, uptake, chlorophyll and H_2O_2 content and enzymatic activity. No signs of necrosis, stunting, chlorosis or wilting were found, while variations were observed concerning physiological and biochemical responses in terms of plant growth, chlorophyll content and induction of reactive oxygen species (ROS). Li et al. (2015) observed that *Arachis hypogaea* seeds exposed to nZVI (0.0024 and 0.0048 mg/L) produced significantly longer seedling compared to negative controls suggesting that nanoparticles may have penetrated the peanut seed coat increasing the water uptake and thus stimulating germination.

This short overview indicated that phytotoxicity data about nZVI are still scarce on macrophytes that are key direct biological targets in case of nanoremediation activities, thus, not sufficient for a sound environmental hazard assessment and most data are based just on nominal concentrations. The aim of this research was to understand the potential effect of nZVI compared to its ionic and micro-sized form considering three well-known testing species (*Lepidium sativum*, *Sinapis alba* and *Sorghum saccharatum*) (Baudo, 2012) and four endpoints (germination, seedling elongation, germination index and biomass).

2. Materials and methods

2.1. Materials and reagents

Commercially available materials were purchased for the experiments: $FeCl_3 \cdot 6H_2O$ (iFe) (Sigma-Aldrich, USA), micro-sized iron (mFe) (Aldrich Chemistry, Germany) and nano-sized zerovalent iron (nFe) (American Elements, USA). Boric acid (Sigma-Aldrich, USA) was used as reference toxicant. Concentrated HCl (34–37%, SpA) and HNO_3 (67–69%, SpA) were purchased from Romil. All reagents used during the experiment were of analytical grade.

Stock solutions and suspensions of 10 g/L and all treatments including negative and positive controls were made in ultrapure water (Zeneer Power III Human 18.3 MΩ cm). Suspensions were sonicated for 1 h at 335 W in an ultrasonic bath (3510 MTH, Branson) just after their gravimetric preparation. Treatment solutions and suspensions were aged 72 h and manually shaken for 1 min before starting phytotoxicity tests.

2.2. Primary and secondary characterisation

Particle size distributions (hydrodynamic diameters) and zeta potential of nFe water suspensions were measured on a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) equipped with a 633 nm HeNe laser operating at 25 ± 0.1 °C in a back-scattering configuration. The suspensions were analysed immediately after their preparation and after 24 h of ageing. The analytical concentration was achieved by mass spectrometry with inductively coupled plasma source (NexION 300D Perkin-Elmer, ICP-MS). Samples for ICP-MS were treated using EPA Method 3051A (EPA, 2007). Briefly, 0.5 g of solution or suspension was weighed in a Teflon vessel followed by the addition of 9 mL of HNO_3 and 1 mL of HCl. Samples were digested with a microwave system (MARS V CEM) according to the following program: temperature ramp up to 175 °C in 5.5 min and maintaining this temperature for 4.5 min. Once cooled, the samples were transferred to 50 mL polyethylene tubes and brought to a final volume of 50 mL with ultrapure water. The instrumental measurement of the obtained solutions was performed in Dynamic Reaction Cell (DRC) mode using NH_3 as reaction gas and Rh at 10 μg/L was used as internal standard.

Treatments were monitored for pH and Eh at 25 °C to keep germination condition under control ($pH > 5$) (OECD, 2006) and evaluate the abundance and partitioning of Fe ionic species. A solution of NaOH 3 M was used to adjust pH levels of iFe treatments above this threshold.

2.3. Ecotoxicity

Phytoxicity tests were carried out according to Beltrami et al. (1999) and OECD (2006). A battery of three macrophytes was selected including two dicotyledonous (*Lepidium sativum* and *Sinapis alba*) and one monocotyledon (*Sorghum saccharatum*) species (Baudo, 2012). Certified seeds were purchased from Ecotox Ltd. (*L. sativum*-lot LES290311; *S. alba*-lot SIA051011; *S. saccharatum*-lot SOS140611). Germination (%), seedling elongation inhibition (SEI, %), germination index (GI, %) (Beltrami et al., 1999) and inhibition of biomass production normalised to germinated seeds (g, dry weight) were considered as endpoints. All endpoints were assessed in triplicate, otherwise explicitly indicated, including negative (ultrapure water) and positive (H_3BO_3) controls. The common accepted threshold level when ten seeds are exposed per replicate in negative control is $\leq 10\%$ (Beltrami et al., 1999; OECD, 2006). The GI can assume values greater or lower than 100%, where a value equal to 100% means that the seedling average length and germination rate between a specific treatment and the