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Biological effects of four iron-containing nanoremediation materials on the green alga *Chlamydomonas* sp.

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ABSTRACT

As nanoremediation strategies for in-situ groundwater treatment extend beyond nanoiron-based applications to adsorption and oxidation, ecotoxicological evaluations of newly developed materials are required. The biological effects of four new materials with different iron (Fe) speciations ([i] FerMEG12 - pristine flake-like milled Fe(0) nanoparticles (nZVI), [ii] Carbo-Iron® - Fe(0)-nanoclusters containing activated carbon (AC) composite, [iii] Trap-Ox® Fe-BEA35 (Fe-zeolite) - Fe-doped zeolite, and [iv] Nano-Goethite - 'pure' FeOOH) were studied using the unicellular green alga *Chlamydomonas* sp. as a model test system. Algal growth rate, chlorophyll fluorescence, efficiency of photosystem II, membrane integrity and reactive oxygen species (ROS) generation were assessed following exposure to 10, 50 and 500 mg L^{-1} of the particles for 2 h and 24 h. The particles had a concentration-, material- and time-dependent effect on *Chlamydomonas* sp., with increased algal growth rate after 24 h. Conversely, significant intracellular ROS levels were detected after 2 h, with much lower levels after 24 h. All Fe-nanomaterials displayed similar Z-average sizes and zeta-potentials at 2 h and 24 h. Effects on *Chlamydomonas* sp. decreased in the order FerMEG12 > Carbo-Iron® > Fe-zeolite > Nano-Goethite. Ecotoxicological studies were challenged due to some particle properties, i.e. dark colour, effect of constituents and a tendency to agglomerate, especially at high concentrations. All particles exhibited potential to induce significant toxicity at high concentrations (500 mg L^{-1}), though such concentrations would rapidly decrease to mg or µg L^{-1} in aquatic environments, levels harmless to *Chlamydomonas* sp. The presented findings contribute to the practical usage of particle-based nanoremediation in environmental restoration.

1. Introduction

Iron (Fe)-based materials possess remarkable potential for the remediation of soil aquifers, groundwater and cyanobacterial blooms (Bardos et al., 2015; Ribas et al., 2016; Sharma et al., 2016). Numerous in-situ applications of zero-valent iron (ZVI) nanoparticles have proved a powerful tool in the clean-up of chlorinated ethenes and toxic metal ions due to their high reductive capacity (Köber et al., 2014; Mueller et al., 2012). Further, emerging particulate materials containing Fe as Fe(0), Fe(II) and Fe(III), where the Fe species act as reductants or sorbents for metals and metalloids, have been used successfully in microbiological contaminant degradation or as heterogeneous Fenton catalysts (Bardos et al., 2015; Mackenzie et al., 2016; Gillies et al., 2017).

Abbreviations: AC, activated carbon; DLS, dynamic light scattering; FCM, flow cytometry; FE SEM, field-emission scanning electron microscopy; FU, fluorescence units; nZVI, nanoscale zero-valent iron; ORP, oxidative reductive potential; PI, propidium iodide; PSII, photosystem II; QY, quantum yield; ROS, reactive oxygen species; ZVI, zero-valent iron

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The overall impact of materials containing Fe(0) on aquatic ecosystems (introduced intentionally or accidentally) remains questionable (Bardos et al., 2015). Other nanomaterials also have the potential to seriously affect aquatic microorganisms such as microalgae, primary producers that play a key role in healthy ecosystems (Adeleye et al., 2016; Klaine et al., 2008). While iron is an essential nutrient in small amounts, increased loading of Fe(II)/Fe(III) ions can rapidly accumulate in the cells of aquatic organisms, resulting in oxidative stress due to the generation of oxide and hydroxide radicals via the Fenton reaction (Crane and Scott, 2012; Davies et al., 2000; Franqueira et al., 2000; Gillies et al., 2016). Moreover, ZVI particles show a strong affinity for cell surfaces; thus, they have the potential to physically damage bacterial or algal cells (Auffan et al., 2008; Lei et al., 2016).

A number of Fe-containing materials have been developed under the European FP7 project NanoRem (for more information see nanorem.eu) in order to provide new and improved materials for treatment of contaminated environments from a broader contaminant spectrum and to offer improved cost effectiveness and safety during transportation and application (Bardos et al., 2015). Up to now, nanoremediation using in-situ generation of permeable reactive barriers or zones through particle subsurface injection has been dominated by nanoiron-based materials. With the introduction of particles with different abilities, nanoremediation has been extended to support bioremediation, advanced oxidation and sorption-assisted clean-up strategies in permeable barriers.

During large-scale in-situ applications, such as those reported for ZVI injection for the treatment of chlorinated organic contaminants (Mueller et al., 2012; Soukupova et al., 2015), suspensions containing up to 10 g L^{-1} of particles are typically injected. Following migration of in-situ applied nanoscale ZVI (nZVI) suspensions within the treated aquifer or water body, Fe concentrations are expected to decline to mg L^{-1} levels or lower (Mueller et al., 2012); hence, ecotoxicological studies should be aimed at such concentrations.

The present study attempts to assess the biological effects of such Fe-containing materials on an aquatic microorganism commonly found in fresh water and soils, *Chlamydomonas* sp., using multiple biological end-points, i.e. growth rate, chlorophyll fluorescence, photosystem II (PSII) quantum efficiency, membrane integrity and intracellular reactive oxygen species (ROS) generation. The algal system was chosen as it is usually associated with contact effects to the cell wall rather than particle incorporation. In addition, behaviour of the Fe-containing materials in the exposure medium was characterised in terms of size, zeta-potential and effect on pH and oxidative reductive potential (ORP).

2. Material and methods

2.1. Fe-containing materials

Four newly developed Fe-containing materials intended for subsurface application as suspensions were examined (see Table 1 for particle descriptions and an overview of their constituents and intended use). The materials were received as dry powders and suspended according to the producers' instructions.

FerMEG12 are metallic ZVI particles that are produced mechanically using a two-stage top-down process and are one of the emerging particles for in-situ groundwater reduction (Köber et al., 2014). Particles of < 40 μm were first generated by dry milling and then more finely ground by wet milling in bivalent alcohol. The milling process forms nanostructured flake-shaped particles.

Carbo-Iron® is a composite of ZVI-nanostructures embedded in activated carbon (AC) particles of about 1 μm. Carbo-Iron® was synthesised carbothermally following a wet impregnation step, where the pores of the colloidal AC particles are filled with ferric nitrate (Fe $(NO_3)_3$) (Bleyl et al., 2012). Electron microscopy following reduction indicates nZVI clusters of predominantly $d_{Fe} \approx 50$ nm built into the AC grain (Mackenzie et al., 2012).

Fe-zeolites is a porous Fe-exchanged alumosilicate mineral particles of the beta-zeolite type with 1.3 wt% total Fe (Gillies et al., 2017) that catalytically activate oxidising agents such as hydrogen peroxide (H_2O_2) (Gonzalez-Olmos et al., 2013). With a specific surface area of 602 m^2 g^{-1} (N_2-BET) and a water-filled pore effective density of $\rho \approx 1.7$ g cm^{-3}, the particles show favourable sedimentation behaviour (i.e. 11–15 mm h^{-1}) for in-situ application (Gillies et al., 2016, 2017).

Nano-Goethite is produced using an industrial FeOOH precursor that undergoes ultrasonification and coating with a layer of a natural organic polymer that results in electro-steric stabilisation (Bosch et al., 2010; Braunschweig et al., 2013). A stable stock suspension of 100 g L^{-1} Nano-Goethite with a mean particle size of 400 nm can be generated in this way.

The shape and particle size of the Fe-containing materials were determined using a Zeiss Ultra Plus field-emission scanning electron microscope (FE SEM). Samples were fixed to aluminium stubs using double-sided carbon tape and cleaned with RF plasma (Evactron) for 10 min before image acquisition. For further details, see Supporting information Fig. S1.

Suspension stabilisers are added in order to generate suspensions stable enough to be injected without major agglomeration (Table 1), carboxymethyl cellulose being added to the FerMEG12 and Carbo-Iron® suspensions and a humic-acid coating used for Nano-Goethite. No stabiliser was added to Fe-zeolites, as it forms a stable suspension.

2.2. Characterisation of Fe-containing materials in the algal exposure medium

The hydrodynamic diameter of each particle type was determined at a range of suspension concentrations (10, 50 and 500 mg L^{-1}) in algal growth medium through dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK) with a 633 nm laser source and a detection angle of 173°. The same instrument was used to measure electrophoretic mobility, which was subsequently transformed to zeta potential using Smoluchowski's approximation. Each sample was measured in triplicate at 30 s intervals. At the beginning and end of each toxicity test, ORP and pH were measured using a standard multimeter (WTW, Germany).

2.3. Algal cultures and exposure conditions

The *Chlamydomonas* sp. used in this study (originally isolated from the Lipno reservoir, Czech Republic) was obtained from the Biology Centre of the Czech Academy of Sciences. The algae were cultivated in Guillard-Lorenzen medium (Guillard and Lorenzen, 1972) (Table S1) in an incubator (PlunoTech, Czech Republic) with a 150 rpm shaker and temperature set to 22 ± 2 °C, applying a light: dark regime of 16:8 h with light intensity set to 1200 lux. The culture was harvested during its exponential growth phase and re-suspended in the exposure media to a cell density of 1×10^6 cells mL^{-1}.

Based on preliminary experiments, where 5 mg L^{-1} of Fe-containing material showed no effect and 1000 mg L^{-1} interfered with measurement, toxicological effect was assessed through exposure to 10, 50 and 500 mg L^{-1} for 2 and 24 h. The experiments were carried out in fully light-transmitting plastic vials containing 5 mL of *Chlamydomonas* sp. and the particle suspension. Negative controls without particles were run in parallel. Exposure experiments were performed under the same conditions (light, temperature and agitation regimes) as those described for the stock algal culture. Possible effects due to shading and particle sedimentation were also considered, particularly as high material concentrations produced a dark (FerMEG12 and Carbo-Iron®; Figs. S2 and S3), skimmed milk-like (Fe-zeolites; Fig. S3) or light brown-red (Nano-Goethite; Fig. S3) suspension. After 2 h and 24 h exposure, 250 μL sub-samples were taken and examined through flow cytometry (FCM) in order to assess algal cell number, cellular membrane integrity and chlorophyll fluorescence. The effect on the ROS generation and