Taking Nanotechnological Remediation Processes from Lab Scale to End User Applications for the Restoration of a Clean Environment

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WP5:
Environmental Impact of Reactive Nanoparticles

DL-5.1:
Dose reponse relationship, Matrix effects on Ecotox

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Executive summary

As innovation in remediation technology brings new solutions to old problems, regulatory bodies require hazard and safety data as new technologies are brought from lab-scale to real-world application. NanoRem has therefore a full work package dedicated to measuring the environmental impact of the nanoparticles used in the project. This is important to ensure that the technology is environmentally safe and that the environmental and societal benefits of removing hazardous pollutants using nanoremediation are not outweighed by the potential hazardous effects of nanoparticles.
The present deliverable compiles the results from ecotoxicity tests on a range of nanomaterials developed during the project. A suite of standard and non-standard ecotoxicity tests were carried out using aqueous suspensions of nanomaterials in the absence of environmental matrices (i.e. soil-free, DOC-free). In the event that nanomaterials exhibited toxicity, we also determined whether the presence of organic matter affected the outcome of ecotoxicity tests (matrix effects on ecotox).

The outcome of ecotoxicity testing in the absence of environmental matrices is summarized in the table below.

EC_{50} >100 mg/L | EC_{50} <100 mg/L | ND: not determined

	P.subcapitata	*Chlamydomonas sp.*	*D. magna*	*L. variegatus*	*V. fischeri*	*E. coli*	*E. fetida*	*L.multiflorum*	*R. sativus*
	48 h growth OECD 201	48 h photosynthesis efficiency	48 h immobilization OECD 202	96 h mortality	15 min luminescence ISO 11348-3	6 h growth 24 h viability	48 h mortality OECD 207	6 day root elongation OECD 208	6 day root elongation OECD 208
NanoFer STAR (NanoIron)									
Nano-magnetite (UPOL)									
Milled Fe particles (UVR-FIA)	ND	ND	ND				ND		
Nano-goethite (HMGU)									
Fe Zeolite (UFZ)									
Carbo-Iron (UFZ)									
Bio-nanomagnetite (UMAN)	ND	ND	ND	ND	ND	ND		ND	ND

The low toxicities found in the standard organisms do not lead to any hazard classification according to EU regulation for any of the tested particles and the results indicate that the particles, except the milled Fe particles, can be considered non-toxic.

In the absence of intrinsic toxicity of most of the particles, the second part of the deliverable was limited to tests on milled Fe particles. The presence of humic acid did not change the outcome of the ecotox tests, contrary to what was expected.

1 Introduction

NanoRem has a full work package dedicated to measuring the environmental impact of the nanoparticles used in the project. This is important to ensure that the technology is environmentally safe and that the environmental and societal benefits of removing hazardous pollutants using nanoremediation are not outweighed by the potential hazardous effects of nanoparticles.

During the first part of the project, the role of WP5 has been to assess the intrinsic toxicity of the various nanoparticles developed by the project partners, in order to contribute to the hazard assessment required under REACH.

The aim of the deliverable DL-5.1 was

1) To establish a baseline for potential (maximum) toxicity of pristine particles in aqueous suspensions. Dose-response relationships were derived from standardized tests where terrestrial and aquatic species were exposed to aqueous suspensions of NPs in the absence of environmental matrices (soil-free, DOC-free, etc.).

2) In the event that nanoparticles exhibited toxicity in the above-mentioned task, to determine whether the presence of organic matter affect the outcome of ecotoxicity tests (matrix effects on ecotox).

2 Ecotoxicity test protocols

2.1 Ecotoxicity tests on aquatic species

2.2 Algal growth test (OECD 201)

The test procedure for the algal growth inhibition tests was the OECD test guideline 201 (OECD, 2011) using the green algae *Pseudokirchneriella subcapitata* as test species in a closed air-tight flask enriched with 1 % carbon dioxide headspace. The OECD algal medium was used to prepare dilution series of the tested materials and samples. All concentrations and controls were then inoculated with an algal culture in exponential growth phase to a density of 5×10^4 cells per mL. The increased cell density, compared to the 10^4 cells per mL recommended in the OECD guideline 201, was intended to increase the signal/noise ratio in the biomass determinations, i.e. differentiate algal pigment fluorescence from background noise resulting from particulate matter and colour of the test compounds. All test concentrations were tested in triplicates and ten controls were used, containing only pure test medium and algae inoculums. Glass vials (20 mL) with algae and test solutions (4 mL) were incubated for 72 h. The containers were placed on a shaker (200 rpm) at 20 ± 2°C and continuously illuminated at 90-110 µE m^{-2} s^{-1} (measured under the test vessel). The light source in the tests was a cold light fluorescent tube emitting light in the visible spectrum (Phillips TL-D 30W/33-640 SLV). Light intensity in the test setup was measured using a LI-COR light meter (model LI-189) with an attached quantum sensor, measuring light within the wavelength range 400-700 nm. The tests were conducted at a pH of 7.0 and measures were taken to avoid that the pH changes more than 0.1-0.2 units during the 72 h exposure period. Samples of 0.4 mL were taken at times 0, 48 and 72 h with a syringe and the algal growth rates calculated on the basis of biomass quantified by acetone extractions of chlorophyll, as described by Mayer et al. (1997). The fluorescence of the samples was measured on a fluorescence spectrophotometer using an excitation wavelength of 430 nm and emission wavelength of 671 nm.

Growth rates and concentration-response curves were estimated by use of a nonlinear regression program (Christensen et al., 2008) assuming log-normal distribution. By use of logistic curve fitting EC-values were determined with corresponding 95 % confidence limits.

2.2.1 Algal assimilation test

The 2-hour ^{14}C-bicarbonate assimilation test with *Pseudokirchneriella subcapitata* (Sørensen & Baun, 2014) was chosen to assess the influence of humic acid on the acute effects of the particles. Earlier algae tests with long-term exposure indicated acute effects with little chronic impact on algae growth, as the algae recovered quickly after an initial reduction in biomass. The tests were carried out in ISO 8692 medium in 20-mL glass scintillation vials fitted with airtight screw caps. In the start of the test 50 µL of a 20 µCi/mL $H^{14}CO_3$-solution (DHI, Hørsholm, Denmark) was added to each of the test vials. After 2 hours of incubation the tests were terminated by addition of 0.4 mL 1% HCl to all vials to evaporate excess $H^{14}CO_3$. 10 mL scintillation liquid (Optiphase "Hisafe" 3, Perkin Elmer, Waltham, MA, USA) was added to each vial, which were then shaken until the mixture was homogeneous and the vials were left in the dark for at least 8 hours before the radioactivity was counted by liquid scintillation counting (TRI-CARB 1600 TR Liquid Scintillation Analyzer, Packard, Meriden CT, USA). The assimilation of ^{14}C was then used as endpoint to assess the impact of the nanoparticles.

2.2.2 Algal photosynthesis efficiency test

Green alga *Chlamydomonas* sp. was provided by the Institute of Hydrobiology, České Budějovice, Czech Republic. The culture was cultivated in Guillard-Lorenzen medium (WC) in a light box with a stable day/night cycle (16 hours/8 hours), temperature was set at 22 ± 2°C. The culture was used when it reached its exponential growth phase. The density of algal cells was then adjusted to 5×10^4 (cells/mL) using UV-vis spectrophotometer (Hach-Lange DR6000, Germany). The culture was exposed to nanoparticles at the following final concentrations: 0 (control), 50, 500 and 1000 mg/L. All tests were carried out in 30 mL of WC medium in glass flasks and incubated in a light box. Aliquots (2.2 mL) of each variant were taken at 0, 24 and 48 hours to detect quantum yield (QY) of photosystem II (PS II). Magnetic nanoparticles were removed right before the measurement using a strong magnet. All measurements were done in duplicates and experiments were repeated three times to ensure data reproducibility. The photosynthesis efficiency was detected using a fluorometer AquaPen-C AP-C 100 (PSI Ltd, Brno, Czech Republic).

QY is a ratio between maximal fluorescence (Fm) emitted by PS II after a very strong and brief light signal that blocks electron transport of the dark phase of photosynthesis, and variable fluorescence (Fv), expressed as Fm minus measured fluorescence after given light signal (F_0).

$$QY = \frac{Fm - F_0}{Fm}$$

Note: the highest concentration of reactive Carbo-Iron, aged Carbo-Iron and activated carbon tested was 100 mg/L instead of 1000 mg/L. This is because test suspensions of Carbo-Iron were too dark at concentrations above 100 mg/L to allow detection of QY of PS II using AquaPen.

2.2.3 Daphnia immobilization test (OECD 202)

The acute toxicity test on daphnids was conducted according to the OECD test guideline 202 (OECD, 2004), using M7 medium as dilution water in the test and for the cultivation of test organisms. For all tests, 3,5-dichlorophenol was used as a positive control for the inhibition of the mobility of *Daphnia magna*. This was done in order to verify the sensitivity of *D. magna*.

The tests were conducted using four replicates per test concentration and five replicates in the control group. Each replicate contained five juvenile *D. magna* in 25 mL of test solution. During the test, the replicates were kept at 20°C ± 2°C. Mobility of the animals was observed after 24 and 48 hours of exposure. Oxygen concentration and pH were measured after 24 and 48 hours in the control replicate and in the highest test concentration.

The pH of the dilution water (in this case M7 media) was within the range 7.8 ± 0.2 and the oxygen concentration had reached saturation. At the end of the test the dissolved oxygen concentration was >2 mg/L and the percentage of immobilization of the controls < 10%. Test results were quantified in terms of EC10- and EC50-values and corresponding 95% confidence intervals estimated by Probit analysis.

2.2.4 Lumbriculus variegatus mortality test

The freshwater oligochaete *Lumbriculus variegatus* was obtained from Aquatic Research Organisms (New Hampshire, United States of America) and kept at 20˚C in US EPA synthetic moderately hard reconstituted water and cultured as described in OECD test guideline 315 (OECD, 2008). The worms were synchronized 10-14 days prior to testing. For every concentration, 10 worms were exposed to 25 mL in separate 50 mL beakers and pH and oxygen levels were measured at the start and the end of the test (96 hours). Mortality was observed every 24 hours as lack of movement after gentle physical stimulus. To assess the lethality of oxygen deprivation, worms were placed in deoxygenated media in airtight bottles for 96 hours and the mortality was observed as described above.

2.3 Ecotoxicity tests on bacteria

2.3.1 Bacterial luminescence bioassay (ISO 11348-3)

The aquatic toxicity test with luminescent bacteria was carried out according to the ISO standard 11348-3 (ISO, 2007) using lyophilized *Vibrio fischeri* supplied in the BioTox Kit (Bio-Orbit, Turku, Finland) as test organisms. The saltwater (2% w/w) used as test medium was buffered with 50 mg/L $NaHCO_3$, and the pH adjusted to 7.0 with 1 M HCl. Tests were carried out in 1-mL glass vials using up to 20 concentrations in two replicates and two controls. Reduction in luminescence after 15 min was used as the endpoint. Concentration–response curves and EC values were estimated as described for the algal test.

2.3.2 Escherichia coli growth test

Gram-negative *Escherichia coli* CCM 3954 was obtained from the Czech Collection of Microorganisms, Masaryk University Brno, Czech Republic. The bacterial inoculums were always prepared fresh by growing a single colony overnight at 37°C in a soya nutrient broth (Sigma-Aldrich). The culture was adjusted to 0.01 – 0.02 optical density at 600 nm (OD600) by UV-vis spectrophotometer (Hach Lange DR6000, Germany) right before performing the toxicity test.

The bacterial culture was transferred to 30 mL of soya broth kept in 200 mL conical flasks. Freshly prepared suspensions of nanoparticles were added to the bacterial culture in a range of final concentrations: 50, 500, and 1000 mg/L. Both negative control (only cells in growth media) and positive control (iron nanoparticles in growth media) were run in parallel (Raghupathi et al., 2011). All samples were incubated at 37°C for 6 hours. Sub-samples were taken every 2 hours for optical density measurement at 600 nm by UV-vis spectrophotometer. Each sample was set in duplicates and the experiment was independently repeated three times. The bacterial growth rate (μ, cells/h) was defined by R linear regression of cell density (OD600) versus incubation time (hour). LOEC and/or NOEC were determined according to the OECD guideline 201 (OECD, 2011).

2.3.3 Escherichia coli viability cell test

E. coli viability test followed the same procedure as *E.coli* growth rate test described in 2.2.2. The controls (bacteria in Soya broth medium only) and bacteria with nanoparticle suspensions were incubated at 37°C for 24 hours. Aliquots of each sample were transferred into a glass test tube (1 mL) and centrifuged at 5000 rpm for 5 minutes. The pellet was washed and resuspended in 1 mL of physiological solution (0.85% of NaCl). The resuspended cells were incubated with LIVE/DEAD BacLight kit (L7007, Life technologies) for 15 minutes. The stained sample with bacteria was placed on glass side to take images by fluorescence microscope with excitation 350-602 nm/emission 402 nm. The non-viable and viable cells were counted by ImageJ software. Always 200 cells were inspected to get the percentage of dead cells and this procedure was repeated three times for one sample. The results were analysed by ANOVA, Dunnett's multiple comparisons test.

2.3.4 Clostridium perfringens growth test

Gram-positive soil bacterium *Clostridium perfringens* CCM 4435 was obtained from the Czech Collection of Microorganisms, Masaryk University Brno, Czech Republic. The bacterial inoculums were always prepared fresh before ecotoxicity testing by growing a single colony overnight in an anaerobe basal broth CM095 (Sigma-Aldrich) in anaerobic condition. The culture was adjusted to 0.01 (±0.002) optical density at 600 (OD600) using UV-vis spectrophotometer (Hach Lange DR6000, Germany) right before performing the test. Freshly prepared suspensions were added to 10 mL of bacterial culture to get final concentrations of nanoparticles: 50, 500 and 1000 mg/L. One-mL aliquots were transferred from each sample onto 24-well plates. Controls, bacterial cells in medium, and NP suspension in medium, were run in parallel. All samples were incubated under anaerobic conditions in cell incubator for 24 hours. Optical density of the samples in 24-well plate was measured at 600 nm (OD_{600}) every 2 hours using multi-plate reader (Synergy HT, BioTek, USA). Each sample was prepared in triplicates. The bacterial growth rate (μ, OD_{600}/h) was defined by R linear regression of cell density (OD600) versus incubation time (hour). The results were analysed by ordinary ANOVA, Dunnett's multiple comparison test.

2.4 Ecotoxicity tests on soil species

2.4.1 Earthworm survival test (OECD 207)

The acute contact test (OECD 207, 1984) is a screening test, which involves exposing earthworms to test material on moist filter paper in order to identify potentially toxic substances to earthworms in soil. Test organisms were adult *Eisenia fetida* weighing between 300 and 600 mg, and having a fully developped clitellum. Earthworms were exposed individually in glass vials lined with filter paper to a

concentration range of test materials, with 3 replicates per concentration and 6 controls. The concentration range recommended in the OECD guideline 207 is 0.1, 1, 10, 100, and 1000 µg/cm2, which implies having test suspensions with concentrations up to 35 g/L. This was only feasible with NanoFer STAR and nano-magnetite (UPOL). For the other test materials received as powder (reactive Carbo-Iron, aged Carbo-Iron, activated carbon, and Fe zeolite), the highest concentration tested was dictated by the sample preparation protocol sent by WP3 partners. For test materials received as suspensions (Nano-goethite, biogenic nano-magnetite, with and without 5% Pd), the highest concentration tested was that of the stock suspensions sent by WP3 partners. For the reasons mentioned above, the highest concentration tested was 280 µg/cm^2 for reactive Carbo-Iron, aged Carbo-Iron, activated carbon and carboxymethyl cellulose, 290 µg/cm^2 for the commercial nano-magnetite, 320 µg/cm^2 for nano-goethite, 420 µg/cm^2 for biogenic nano-magnetite, 600 µg/cm^2 for biogenic nano-magnetite with 5% Pd, and 700 µg/cm^2 for Fe zeolite.

Mortality was assessed after 48 h. The test was considered valid if the mortality in control earthworms was not exceeding 10 %. As the guideline was originally intended for dissolved chemicals, some adjustments were necessary for suspended particulate materials. First, certain data regarding the n-octanol/water partition coefficient, water solubility and vapour pressure were disregarded, as they are of limited relevance for particulate materials. Secondly, characterization of the exposure suspensions were carried out as described in IDL-4.1 Standardized experimental protocols for mobility, reactivity and ecotoxicity studies (§2. Characterization of nanoparticles and their aqueous suspensions), including whenever possible: oxidation reduction potential, pH, temperature, total Fe concentration, zeta potential and zeta-averaged hydrodynamic diameter.

2.4.2 Seed germination and root elongation test (OECD 208)

The seed germination/root elongation toxicity test is described in the OECD test guideline 208 (OECD, 2006) and in the EPA guideline OPPTS 850.4200 (EPA, 1996). Briefly, seeds of ryegrass (*Lolium multiflorum*, monocot) and radish (*Raphanus sativus*, dicot) were exposed for 6 days to test materials at 0, 0.01, 0.05, 0.1, 1, 5 and 10 g/L (nominal concentrations), with 3 petri dishes containing 10 seeds each, for each concentration and each plant species.

To avoid any prior contamination before exposure, the seeds were first sterilized by soaking them in H_2O_2 solution (3 %) for 1 min, rinsed several times with distilled water, soaked for about two hours in distilled water and finally blot-dried on filter paper. To avoid low germination rate in the controls, it is important to select only the seeds settled at the bottom of the washing beaker, and to avoid small and discolored seeds. The test was conducted in petri dishes (PE-LD, 15 mm × 100 mm) containing one piece of cellulose filter paper (85 g/m^2, 0.19 mm thick, Quantitative ashless paper grade 589/2, Schleicher and Schuell). Each petri dish received 5 mL of test suspension and 10 seeds, evenly spaced on top of the filter paper. Petri dishes were incubated at 21°C in the dark for 6 days. At the end, pictures of the seedlings were taken and the root length measured using the ImageJ software. Oxidation-reduction potential, pH, zeta-potential and zeta-averaged hydrodynamic diameter were recorded at the beginning and at the end of the experiment. Samples for total Fe concentration analysis by ICP-OES were taken at the beginning of the experiment.

All root length data were analysed using a two-way ANOVA (factors: replicate dish and concentraation), followed by a pairwise multiple comparison test, using the Holm-Sidak method. In addition to this parametric approach, a non-parametric analysis of the data was done when normality and

homoscedasticity assumptions were violated. In this case, a Kruskal-Wallis one-way (factor: concentration) analysis of variance on ranks was run, followed by a pairwise multiple comparison test, using the Dunn's Method.

3 Test materials: sample preparation for toxicity testing

3.1 Particles from WP2

3.1.1 NanoFer STAR (NanoIron)

A stock suspension of NanoFer STAR at 250 g/L was prepared following the instructions sent by NanoIron in the "Manual for preparation of an aqueous suspension from dry stabilized powder NanoFer STAR". For 20 mL of stock suspension at 250 g/L, 20 mL of deionized water was added to 5 g of test material in a falcon tube and mixed for 10 min with a high-shear mixer (e.g. IKA Ultra Turrax T25 with dispersing tool S25N-10G). Dilution series were prepared and used right away.

Note: We learned later that this batch of NanoFer STAR had a low content of ZVI, and was closer to magnetite in composition. Additional tests on more representative batches of NanoFer STAR might be carried out in the future. This is, however, not a priority since the material is already available on the market (we expect that toxicity tests have been carried out and fulfilled the criteria for commercialization).

3.1.2 Nano-magnetite (UPOL)

The precursor of NanoFer STAR, nano-magnetite was received as powder from Palacky University in Olomouc. A stock suspension of nano-magnetite at 250 g/L was prepared and used in exactly the same way as NanoFer STAR.

3.1.3 Milled Fe particles (UVR-FIA)

A sample of milled Fe particles (KKM 03-06, May 2014) was received from UVR-FIA on May 30th 2014. The original suspension had a shiny metallic deposit and was shaken until the suspension was homogeneous. It is worth noticing that the metallic deposit reappeared within seconds after shaking stopped.

According to the safety data sheet sent by UVR-FIA, the product consists of 15-30% iron, 70-85% monoethylene glycol and <0.2% surfactants. The iron concentration in the original suspension is therefore between 150 and 300 mg Fe per g of suspension. Dilution series were made from a 1000 mg/L stock (0.67 g original particle suspension in media), which was sonicated for 15 minutes. A solvent control with monoethylene glycol was also included. For the plant test, dilution series were made from a 10 g/L stock (6.7 g original particle suspension in DI water).

3.2 Particles from WP3

3.2.1 Nano-goethite (HMGU)

An air stable stock suspension of nano-goethite was received from HMGU, with a concentration of approximately 0.7 mol/L FeO(OH) and 39 g Fe/L. Dilution series were prepared directly from this stock suspension.

3.2.2 Fe zeolite (UFZ)

The iron containing zeolite Fe-BEA-35 was received as powder from UFZ. The sample preparation was done in two ways, mainly due to a lack of coordination among partners. However, both sample preparation procedures could find a justification: the first gives information about the material suspended in the way recommended by the supplier, the second gives information about the pristine material.

- Sample preparation for the algal photosynthesis efficiency test, *E. coli* growth test, earthworm survival test, and seed germination/root elongation test:

A stock suspension with a zeolite concentration of 25 g/L was prepared following the instructions sent by UFZ "Standard procedure and setup for preparation of suspensions of the Fe-zeolite material Fe-BEA-35". For 100 mL of stock suspension at 25 g/L zeolite, 2.5 g carboxymethyl cellulose (CMC) in 50 mL deionized water was heated at 70°C and stirred for an hour, while 2.5 g zeolite in 50 mL deionized was sonicated for 15 min. The CMC solution and zeolite suspension were then mixed and sonicated for 15 min. Dilution series were prepared and used right away.

- Sample preparation for algal growth test, *Daphnia* immobilization test, bacterial luminescence bioassay:

Same sample preparation as for NanoFer STAR, CMC was not added to the suspension.

3.2.3 Carbo-Iron and associated (UFZ)

Reactive Carbo-Iron (CIC-130920), aged Carbo-Iron (CIC-a), and activated carbon (AC-01) were received as powder from UFZ. The reactive Carbo-Iron was stored under argon atmosphere to prevent partial oxidation. In case of the aged Carbo-Iron, the zero-valent iron had been oxidized in water to generate a material that represents the particle state after injection into the aquifer. Activated carbon was included in the testing as it is the educt of the composite material Carbo-Iron and could be considered as reference material without iron loading. The sample preparation was done in two ways, for the same reasons as mentioned in §3.2.2.

- Sample preparation for the algal photosynthesis efficiency test, *E. coli* growth test, earthworm survival test, and seed germination/root elongation test:

Stock suspensions of reactive Carbo-Iron, aged Carbo-Iron and activated carbon were prepared as described in the protocol sent by UFZ. For 100 mL of a stock suspension at 10 g/L, 20 mL of a 10 g/L CMC solution was added to 80 mL of test medium and degassed with N2 for an hour. Then, 1 g of test material was added to the solution under N_2 flow, and mixed for 10 min with Ultra Turrax. Dilution series were prepared under regular aerobic conditions and used right away.

- Sample preparation for algal growth test, *Daphnia* immobilization test, bacterial luminescence bioassay:

- Same sample preparation as for NanoFer STAR, CMC was not added to the suspension.

3.2.4 Bio-nanomagnetite (UMAN)

Suspensions of bio-nanomagnetite (Fe_3O_4) and palladised bio-nanomagnetite (5%Pd-Fe_3O_4) were received from the University of Manchester, together with a sample of commercial nanomagnetite (sent as powder) for a comparison with bioengineered nanomagnetite. Due to the limited amount of

samples, bio-nanomagnetite was only tested on earthworms. Tests on bacteria and aquatic organisms will be carried out during the third year of the project.

4 Methods for characterization of exposure suspensions

4.1 Hydrodynamic diameter, size distribution and zeta-potential

Dynamic Light Scattering (DLS) measurements were performed on a Malvern Zetasizer ZS (Malvern instruments Ltd, Worcestershire, UK) equipped with a laser source at a wavelength of 633 nm. Zeta-averaged hydrodynamic diameters and size distributions (when needed) were determined using the "multiple narrow modes (high resolution)" algorithm supplied by Malvern. Measurements were done in triplicates of 5 runs with autocorrelation functions of 10 seconds.

The same instrument was used for the measurements of electrophoretic mobility and the Smoluchowski approximation was used for determining zeta-potentials. Three measurements with 5 runs per measurement were obtained.

NTA measurements of the hydrodynamic diameter of individual particles were done on a Nanosight LM10 (NanoSight Ltd, Amesbury, UK). The light source is a solid-state, single-mode laser diode (radiation output max power <50µW, 635nm continuous wave, max power < 35mW). The standard camera Marlin F-033B (Allied Vision Technologies GmbH, Stadtroda, Germany) was used. All data were analyzed using the instrument software (NanoSight™ version 2.2). The analysis with NTA was done on 7 videos with 1 min length each.

4.2 Oxidation reduction potential and pH

The solution oxidation reduction potential and pH was measured in all exposure suspensions at the beginning and the end of the tests. In order to get reliable pH measurements, it is recommended to calibrate the electrode (at pH 4, 7, and 10) before use, to rinse the electrode thoroughly between each buffer and even more after calibration. The pH measurement of low ionic strength solutions following immersion in a calibration buffer requires special attention: the pH of distilled water should be around 5.7, and the electrode should be rinsed with distilled water until stable readings are achieved. Several measurements should then be made for each suspension, enough to get two similar consecutive values (usually 3 measurements are enough to fulfill this criterion).

4.3 Total iron concentration

Sample digestion with concentrated HCl (37%) overnight at room temperature, followed by filtration at 0.45 µm, was not strong enough to digest all material types. New trials using a microwave digester Discover SP-D are ongoing.

4.4 Electron microscopy

Electron scanning microscopy (JEOL 840 SEM) was used to observe the root of seedlings exposed to Fe zeolite. The seedlings were first let overnight in a fixative made of paraformaldehyde, glutaraldehyde and PIPES buffer (pH 7.2), then sequentially dehydrated in ethanol, and finally dried and coated using a critical point drying instrument.

5 Results

5.1 Results for direct exposure to organisms

5.1.1 NanoFer STAR (NanoIron)

- Algal growth test

No signs of toxicity to *P. subcapitata* were observed at concentrations up to 100 mg/L (data not shown). However, at higher concentrations the particles interfered with chlorophyll measurements, which rendered the assessment of toxicity unfeasible.

- Algal photosynthesis efficiency test

No adverse effects to *Chlamydomonas* sp. were observed at concentrations up to 1000 mg/L, which was the highest concentration tested (Figure 1)

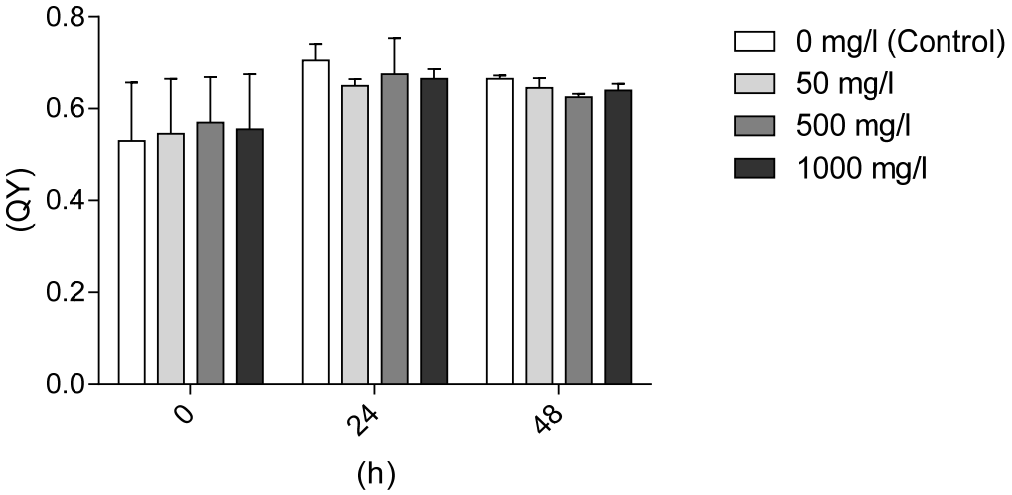


Figure 1 Photosynthesis efficiency (QY) of green alga *Chlamydomonas* sp. exposed to different concentrations of NanoFer STAR

- Daphnia immobilization test

No adverse effects to *D. magna* were observed at concentrations up to 1000 mg/L, which was the highest concentration of NanoFer STAR tested (data not shown).

- Lumbriculus mortality test

No adverse effects to *L. variegatus* were observed at concentrations up to 100 mg/L, which was the highest concentration of NanoFer STAR tested (data not shown).

- Bacterial luminescence bioassay

No signs of toxicity to *V. fischeri* were observed at concentrations up to 100 mg/L (data not shown). However, at higher concentrations the particles interfered with the measurement of luminescence, which made the assessment of toxicity unfeasible.

- E. coli growth test

The EC_{10} was 130 mg/L, the highest concentration tested was 1000 mg/L (data not shown). The pH and ORP of the exposure media are shown in Figure 2.

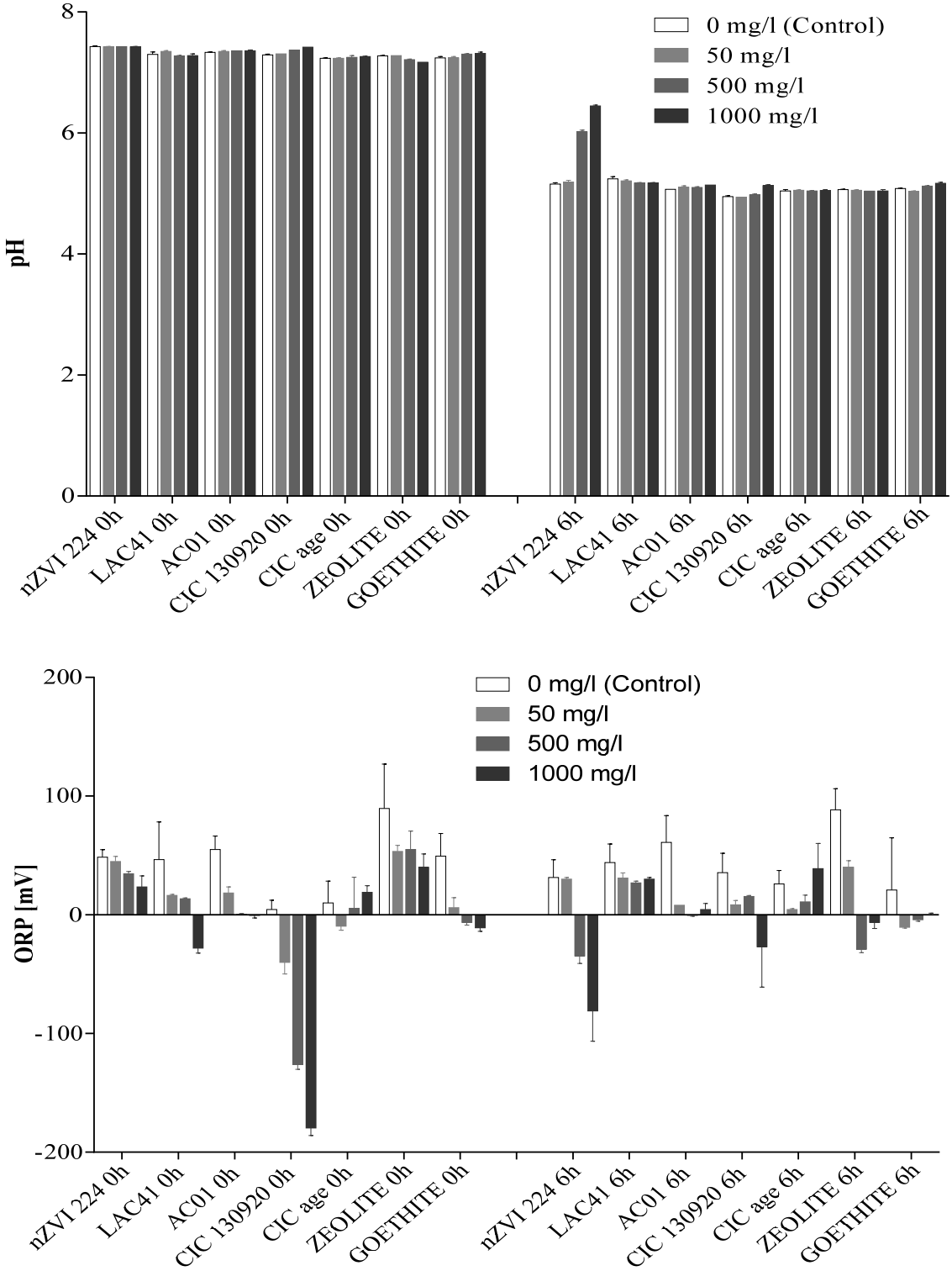


Figure 2 pH (upper graph) and oxidation-reduction potential (ORP, lower graph) in cultivation media with *E. coli* exposed to NanoFer STAR (nZVI 224), Nano-magnetite (LAC41), Nano-goethite (GOETHITE), Fe zeolite (ZEOLITE), Activated carbon (AC01), Reactive Carbo-Iron (CIC 130920), Aged Carbo-Iron (CIC age) at time 0 and 6 hours.

- E. coli viability test

There were only a few dead cells in all tested NanoFer STAR concentrations, not statistically different from control samples (1.7% of dead cells) (Figure 3). The highest NanoFer STAR concentration tested was 1000 mg/L. The pH and ORP of the exposure media are shown in Figure 2.

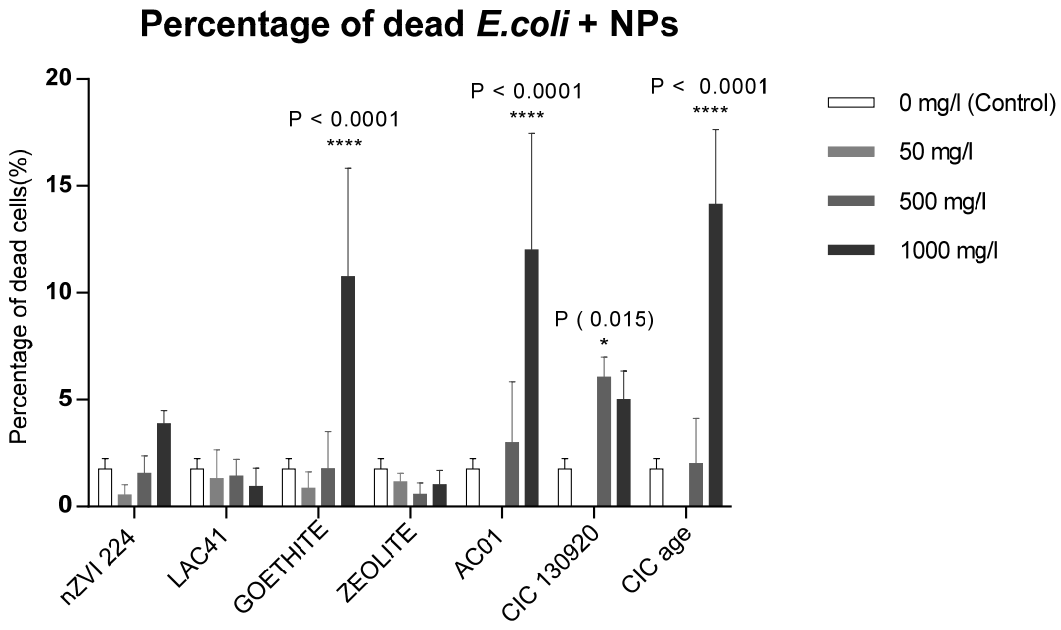


Figure 3 Percentage of dead *E. coli* cells exposed to NanoFer STAR (nZVI 224), Nano-magnetite (LAC41), Nano-goethite (GOETHITE), Fe zeolite (ZEOLITE), Activated carbon (AC01), Reactive Carbo-Iron (CIC 130920), Aged Carbo-Iron (CIC age) for 24 hours. Statistically significant differences are indicated by asterisks (ANOVA; Dunnett's multiple comparisons test)

- Earthworm survival test

No adverse effect was observed at concentrations up to 35 g/L (1000 µg/cm^2), which was the highest concentration of NanoFer STAR tested (data not shown). All earthworms survived and showed no sign of distress.

- Seed germination and root elongation test

Characterization of the exposure suspensions

The measurements of zeta-averaged hydrodynamic diameters with NanoFer STAR measured for samples without seeds in quiescent conditions stored in the DLS cuvettes were inconclusive due to strong sedimentation that occurred within minutes after preparing the different concentrations (see Figure 4). Except for some exceptional cases, measurements done on the supernatant did not provide information about size due to insufficient signal; the samples that were possible to measure presented strong variation and the sizes varied between 1 µm and 20 µm. Parallel to these measurements, zeta potential was also tested on separately stored samples. However, these measurements were also flawed due to the effects of sedimentation, as explained above; however, when it was possible to measure zeta-potentials they were negative and larger than -10mV.

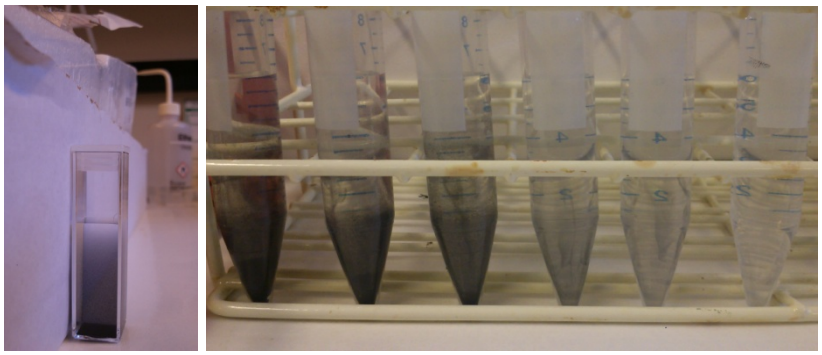


Figure 4 Photo taken on the first day of the suspensions used in the tests. Left photo shows the suspension of 10 g/L NanoFer STAR 2 minutes after strong shaking. Right photo shows the suspensions used for zeta-potential measurements 2 hours after preparing the dispersions. Concentrations tested in the right photo were (from left to right) 10, 5, 1, 0.1, 0.05, 0.01 g/L prepared as specified in the protocols.

Additional measurements were performed on samples run in parallel to the exposure tests which contained double amount of dispersion (10 mL) and 10 seeds of *Raphanus sativus* and were collected in the final day with the aim of evaluating the effects of organic matter produced by the seeds on the particles. The pictures of the petri dish in the last day are presented in Figure 5.

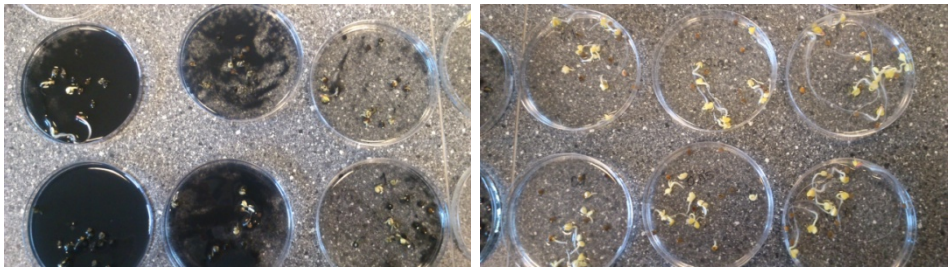


Figure 5 Photo taken on the last day of the suspensions used in the test with particles and seeds. Left photo shows the suspension of NanoFer STAR concentration 10, 5 and 1 g/L. Right photo shows the suspensions concentrations 0.1, 0.05, 0.01 g/L. Two replicates per concentration were evaluated.

After 6 days exposure, DLS zeta-averaged hydrodynamic diameter measured in the liquid collected from the exposure tests with seeds indicated that particles with a size at around 200 nm were present and the zeta potentials also showed values between -8 and -13 mV. The results are presented in Figure 6. Further analysis of the effects of the seeds exudates on these particles is being currently processed. Three weeks after their preparation, the suspensions exposed to the seeds had oxidized extensively with a large part of the settled particles colored red in the concentrations 1, 5 and 10 g/L. In contrast, the particles that were stored in DI water did not present any visible change.

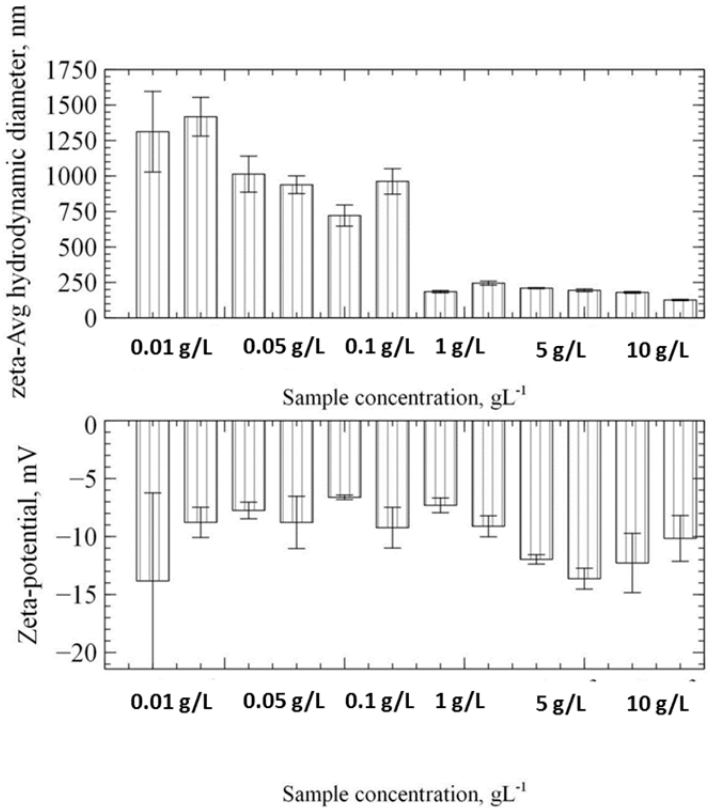


Figure 6 Characterization of aqueous dilutions of NanoFer STAR used in the plant root elongation test, after 6 days. Zeta-averaged diameters obtained from DLS measurements and zeta-potential values obtained from electrophoretic mobility measurements done in parallel to the tests. Averages of three replicated measurements are pre-sented and error bars represent 1 standard deviation.

The pH of the exposure suspensions (same experimental conditions but without seeds) at the beginning and the end of the test was ranging from 6.1 to 6.7. The ORP ranged from 270 mV (control) to 50 mV (10 g/L) on day 1, and from 240 mV (control) to 140 mV (10 g/L) on day 6. The ORP of the 10 g/L suspension was lower than that of the control, but not as low as it should have been if the NanoFer STAR was “fresh”. This result seems to indicate that the material had already lost some of its reducing power.

Note: This confirms what we learned some months later: that the material was closer to magnetite than to ZVI in composition.

Seed germination and root elongation

NanoFer STAR did not affect the germination rate of *Raphanus sativus* and *Lolium multiflorum* (highest concentration tested 10 g/L, results not shown). Root elongation was reduced at concentrations ≥ 1 g/L for *R. sativus*, while no significant reduction in root length of *L. multiflorum* was observed (Figure 7).

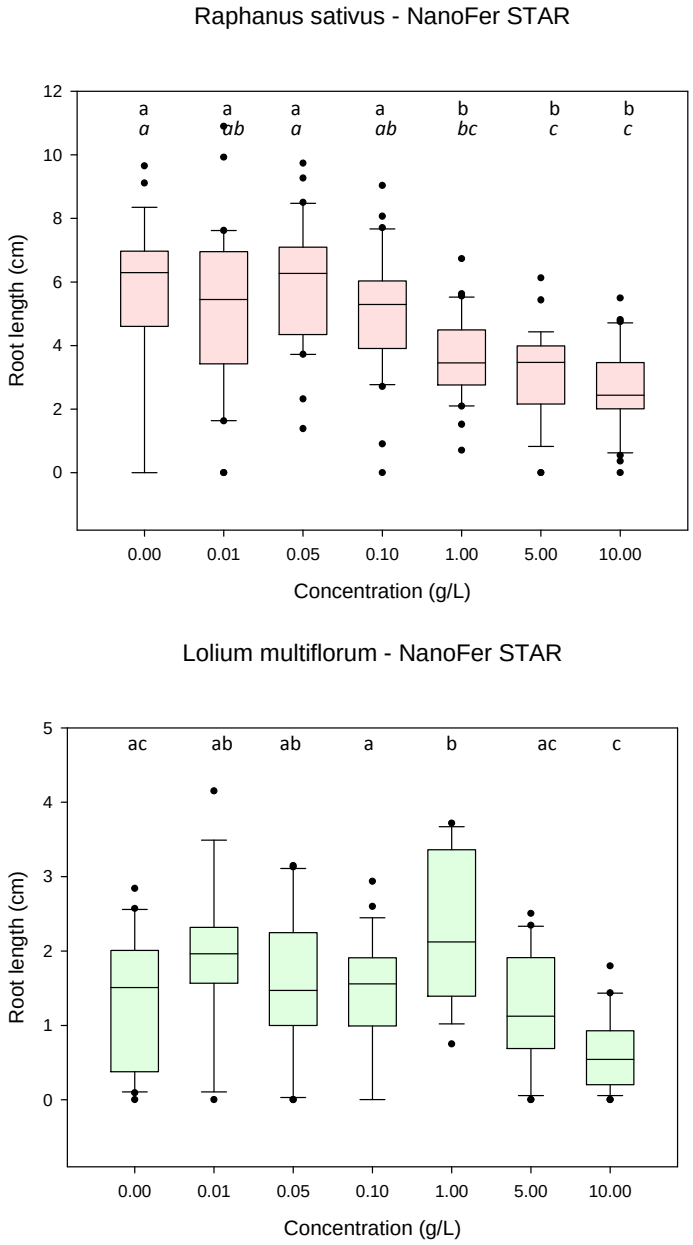


Figure 7 Root length of *Raphanus sativus* and *Lolium multiflorum* exposed to NanoFer STAR for 6 days. Statistically significant differences are indicated by different letters (normal: ANOVA; italics: Kruskal-Wallis ANOVA on ranks)

5.1.2 Nano-magnetite (UPOL)

- Algal growth test

No signs of toxicity to *P. subcapitata* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with chlorophyll measurements, which rendered the assessment of toxicity unfeasible.

- Algal photosynthesis efficiency test

No adverse effects to *Chlamydomonas* sp. were observed at concentrations up to 1000 mg/L, which was the highest concentration tested (Figure 8).

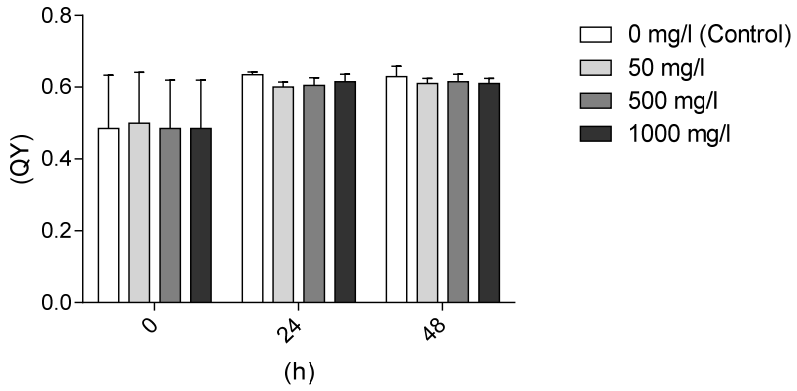


Figure 8 Photosynthesis efficiency (QY) of green alga *Chlamydomonas* sp. exposed to different concentrations of nano-magnetite

- Daphnia immobilization test

No adverse effects to *D. magna* were observed at concentrations up to 1000 mg/L, which was the highest concentration of magnetite tested (data not shown).

- Lumbriculus mortality test

No adverse effects to *L. variegatus* were observed at concentrations up to 100 mg/L, which was the highest concentration of nano-magnetite tested (data not shown).

- Bacterial luminescence bioassay

No signs of toxicity to *V. fischeri* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with the measurement of luminescence, which rendered the assessment of toxicity unfeasible.

- E. coli growth test

The EC_{10} was 225 mg/L, the highest concentration tested was 1000 mg/L (data not shown). The pH and ORP of the exposure media are shown in Figure 2.

- E. coli viability test

There were only a few dead cells in all tested nano-magnetite concentrations, not statistically different from control samples (Figure 3). The highest concentration tested was 1000 mg/L. The pH and ORP of the exposure media are shown in Figure 2.

- Earthworm survival test

No adverse effect was observed at concentrations up to 35 g/L (1000 µg/cm^2), which was the highest concentration of nano-magnetite tested (data not shown). All earthworms survived and showed no sign of distress.

- Seed germination and root elongation test

No adverse effect on the germination rate was observed. The root length was only reduced at 10 g/L (Figure 9).

Figure 9 Root length of *Raphanus sativus* exposed for 6 days to various concentrations of nano-magnetite (from left to right: control, 0.1, 1, and 10 g/L).

5.1.3 Milled Fe particles (UVR-FIA)

- Bacterial luminescence bioassay

The results from the bioassay are presented in Figure 10 and the data shows a clear dose-response relationship as seen in Figure 11. As these particles also showed acute toxicity in the algal growth test (data not shown), shading and other possible test interferences are not suspected to have influenced the test. Based on Figure 11, EC_{10}, EC_{20} and EC_{50} values (and their 95% confidence interval) are estimated to be 76 mg/L (53-108), 156 mg/L (122-200), and 627 mg/L (550-713), respectively. Solvent controls showed no effect compared to media controls.

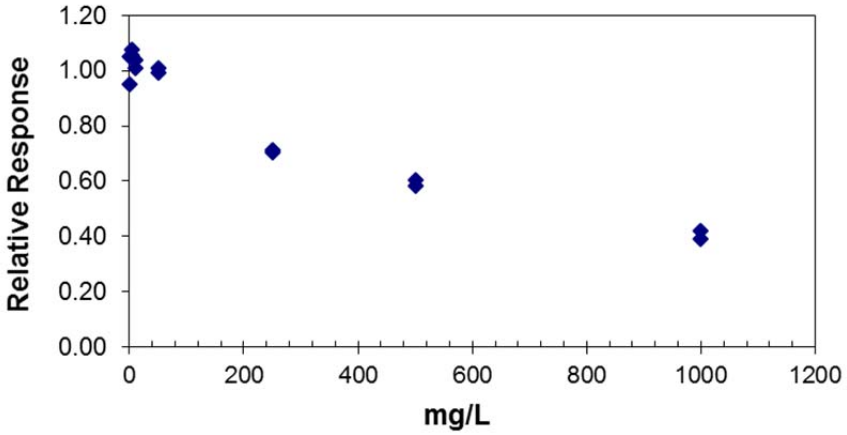


Figure 10 Relative response (luminescence) from *Vibrio fischeri* after 15 minutes of exposure to various concentrations of the milled Fe particles KKM 03-06 compared to media controls. Solvent controls showed no effect.

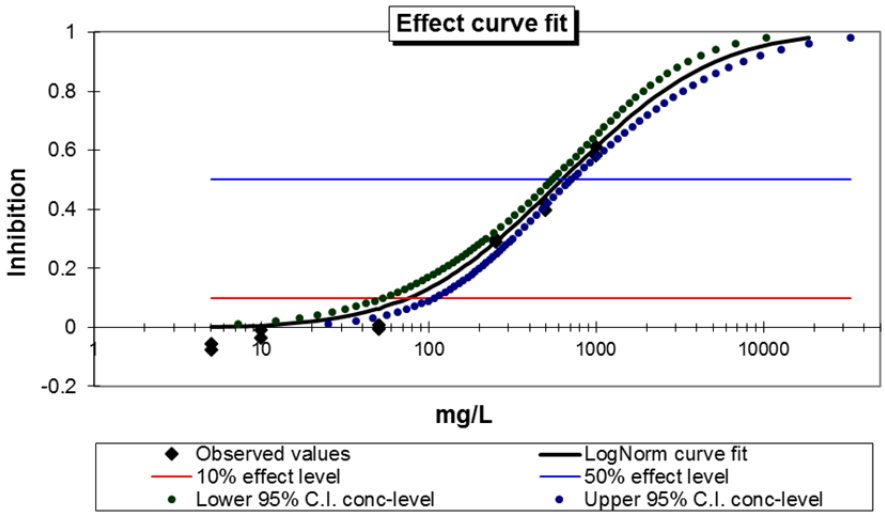


Figure 11 Dose-response curve of the inhibition by milled Fe particles KKM 03-06 of bacterial luminescence (with 95 % confidence interval levels).

- E. coli growth test

After 6h exposure to Fe milled particles KKM 03-06, the growth rate of *E. coli* was 60%, 23%, and 3% of the growth rate in the control at 50, 500, and 1000 mg/L, respectively (Figure 12).

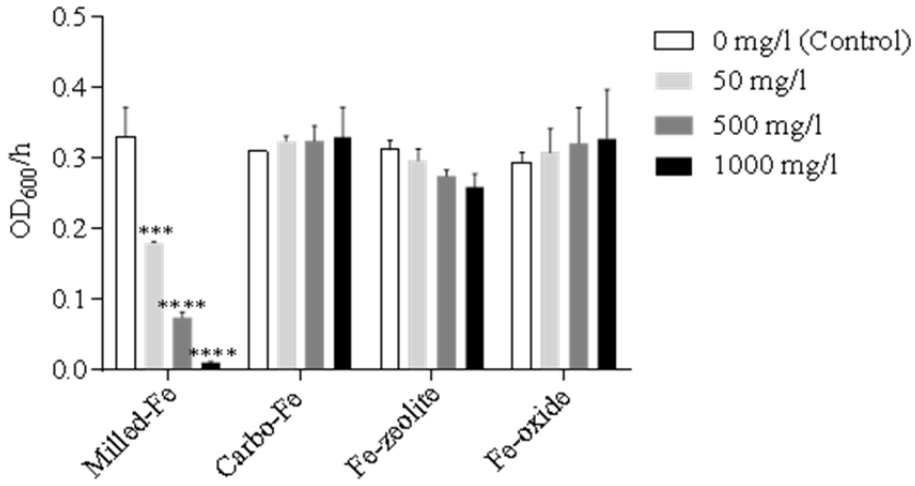


Figure 12 Growth rate of *E. coli* after 6h exposure to milled Fe, Carbo-Iron, Fe-zeolite and Fe-Oxide (nano-goethite) at 0, 50, 500 and 1000 mg/L. P = 0.0003 (***), P < 0.0001 (****)

- E. coli viability test

Cell viability was strongly reduced after 24h exposure to milled Fe particles KKM 03-06 at 500 and 1000 mg/L, as shown in Figure 13.

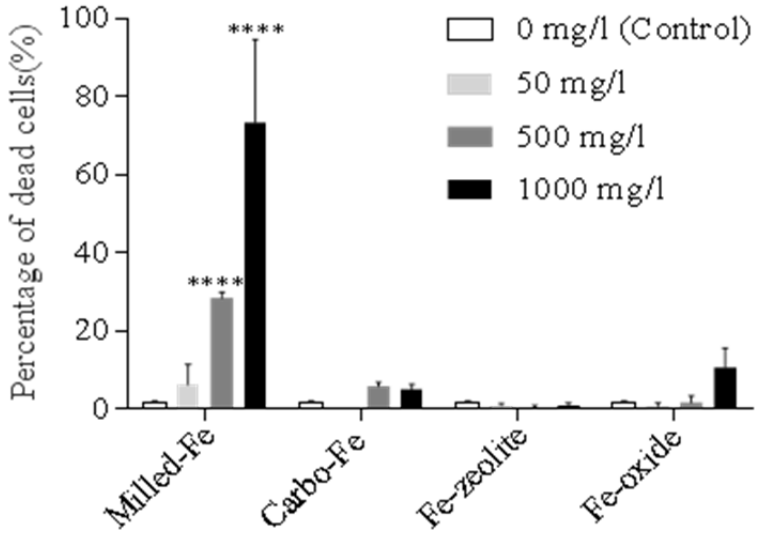


Figure 13 Percentage of dead *E. coli* cells after 24h exposure to milled Fe, Carbo-Iron, Fe-zeolite and Fe-Oxide (nano-goethite) at 0, 50, 500 and 1000 mg/L. P < 0.0001 (****).

- Lumbriculus variegatus mortality test

The milled Fe particles KKM 03-06 proved the most reactive of all the particles tested, with an EC_{50} between 1 and 5 mg/L (see Table 1). The particles also drastically reduced the oxygen content in the test (down to 1.5 mg/L dissolved O_2 in presence of 100 mg/L milled Fe particles). However, further investigations clarified that the observed toxicity was not due to the lack of oxygen.

Table 1. 96 hours *Lumbriculus variegatus* ecotoxicity test with milled Fe particles KKM 03-06.

Milled Fe particles	**# of dead worms out of 10**							
Concentration (mg/L)	**24h**	**48h**	**72h**	**96h**	**pH start**	**Oxygen start (mg/L)**	**pH 96h**	**Oxygen 96h (mg/L)**
1	0	0	0	0	8.2	8.6	8.4	9.3
5	6	7	8	8	8.2	7.4	8.4	9.0
10	10	10	10	10	8.0	5.4	8.3	8.8
50	10	10	10	10	7.9	4.0	8.3	5.0
100	10	10	10	10	8.1	1.5	8.0	3.9
Control	0	0	0	0	8.3	8.7	8.3	8.6

- Seed germination and root elongation test

Characterization of the exposure suspensions

The measurements done with milled Fe particles KKM 03-06 included zeta-averaged hydrodynamic diameters measured on the first and sixth (last) day. Parallel to these, zeta potential measurements were also performed on the same stored samples. Additional measurements of zeta-average diameter and zeta-potentials were performed on samples collected on the last day from the containers containing double amount of suspension and the seeds of Raphanus sativus, but without filter paper.

DLS zeta-averaged hydrodynamic diameter measured for samples (without and with seeds) are presented in Figure 14 (upper graphs). The results show a high variation in size during the whole duration of the test and some degree of stabilization achieved when the particles are in the presence of seeds.

The measurements of zeta-potential are presented in Figure 14 (lower graphs). Selected samples without seeds were kept in the dark during the duration of the test and approximately 1 mL was used to measure the zeta-potentials. On the last day, samples that had been exposed to the seeds were also measured. In general, the zeta-potentials on the last day were more negative in suspensions containing seeds/germlings than in suspensions that had been aged without seeds.

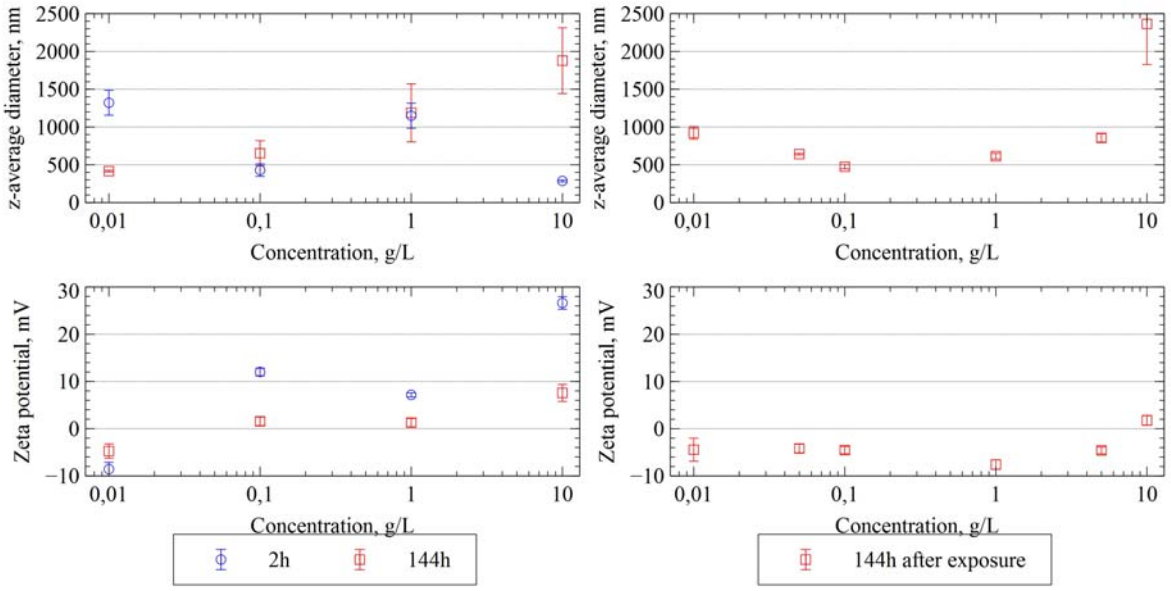


Figure 14 Characterization of aqueous dilutions of milled Fe particles KKM 03-06 used in the plant root elongation test. Zeta-averaged diameters obtained from DLS measurements and zeta-potential values obtained from electrophoretic mobility measurements done in parallel to the tests. The right panel corresponds to measurements performed the last day on exposure suspensions containing seeds/germlings. Averages of three replicated measurements are pre-sented and error bars represent 1 standard deviation.

Additional measurements were performed in NTA of the control and the three lowest concentrations (Figure 15) exposed to seeds. These concentrations were in the range of concentration (number-based) that NTA can handle. The results confirm the presence of material between 200 and 400 nm in these samples. The presence of large particles was not observed when performing the measurements even if they were not avoided intentionally; this suggests that the number of such big particles was not significant in number compared to the small ones.

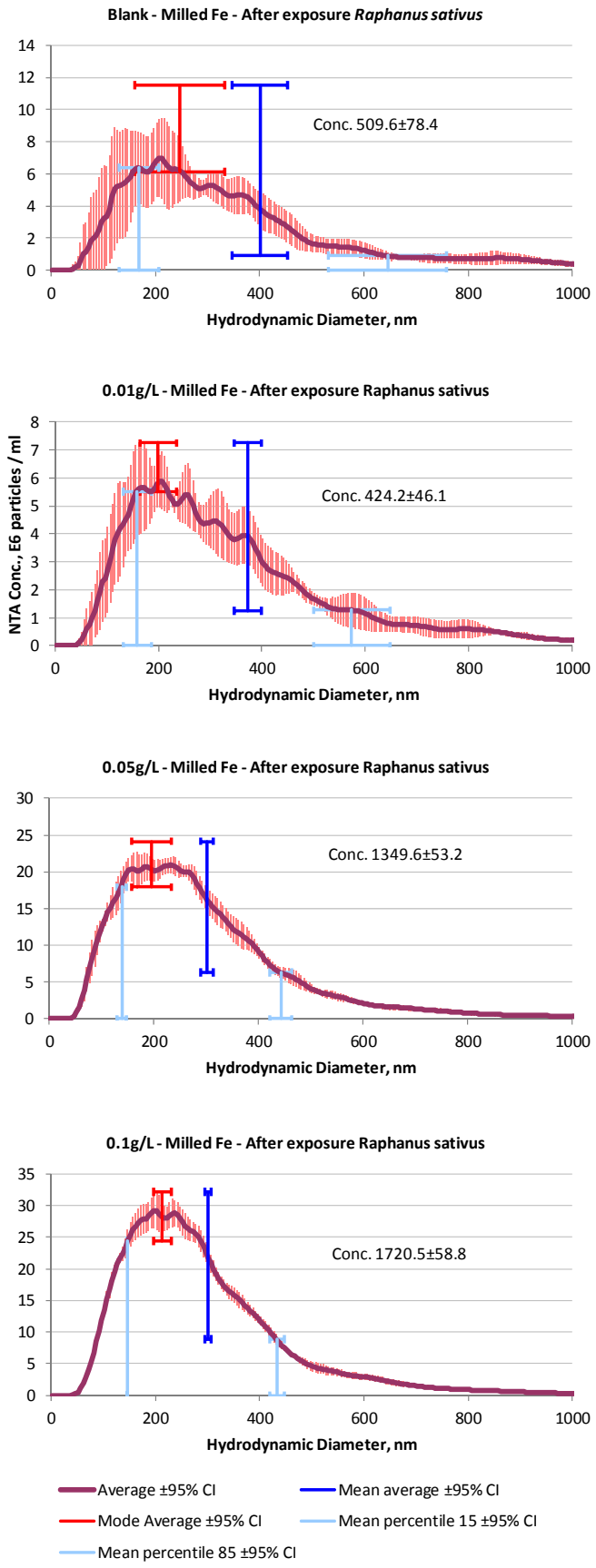


Figure 15 Particle size distribution (PSD) of the samples after exposure to *Raphanus sativus* to milled Fe particles KKM 03-06, with nominal Fe concentrations of 0, 0.01, 0.05 and 0.1 g/L. Complete lines correspond to average values of five videos of 60 s each. Error bars repre-

sent the 95% confidence interval (CI) among the videos. Mean values, mode values, 15th and 85th percentiles were calculated for each video and are presented in the PSD as average values of five videos with the respective 95% CI. Inserts are total number concentrations presented for each sample with the corresponding 95% CI.

The pH of the exposure suspensions (same experimental conditions but without seeds) during the experiment was 6 ± 0.5. The ORP was ranging from +250 mV (control) to -590 mV (10 g/L) at the beginning of the experiment (Figure 16).

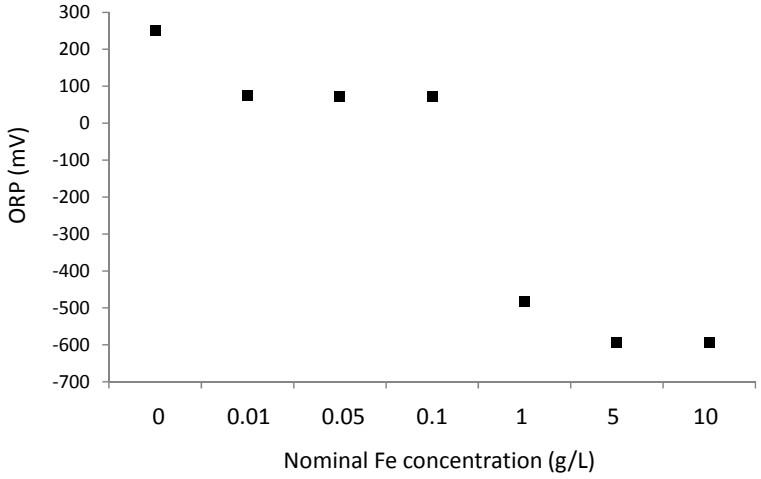


Figure 16 Oxidation reduction potential in aqueous suspensions of milled Fe particles at various nominal Fe concentrations.

Plant root elongation

The root elongation of *Raphanus sativus* was significantly reduced at nominal Fe concentrations of 0.05, 0.1, and 5 g/L, and totally inhibited at 10 g/L (Figure 17, top). The root elongation of *Lolium multiflorum* was significantly reduced at nominal Fe concentrations > 1 g/L, and totally inhibited at 10 g/L (Figure 17, bottom).

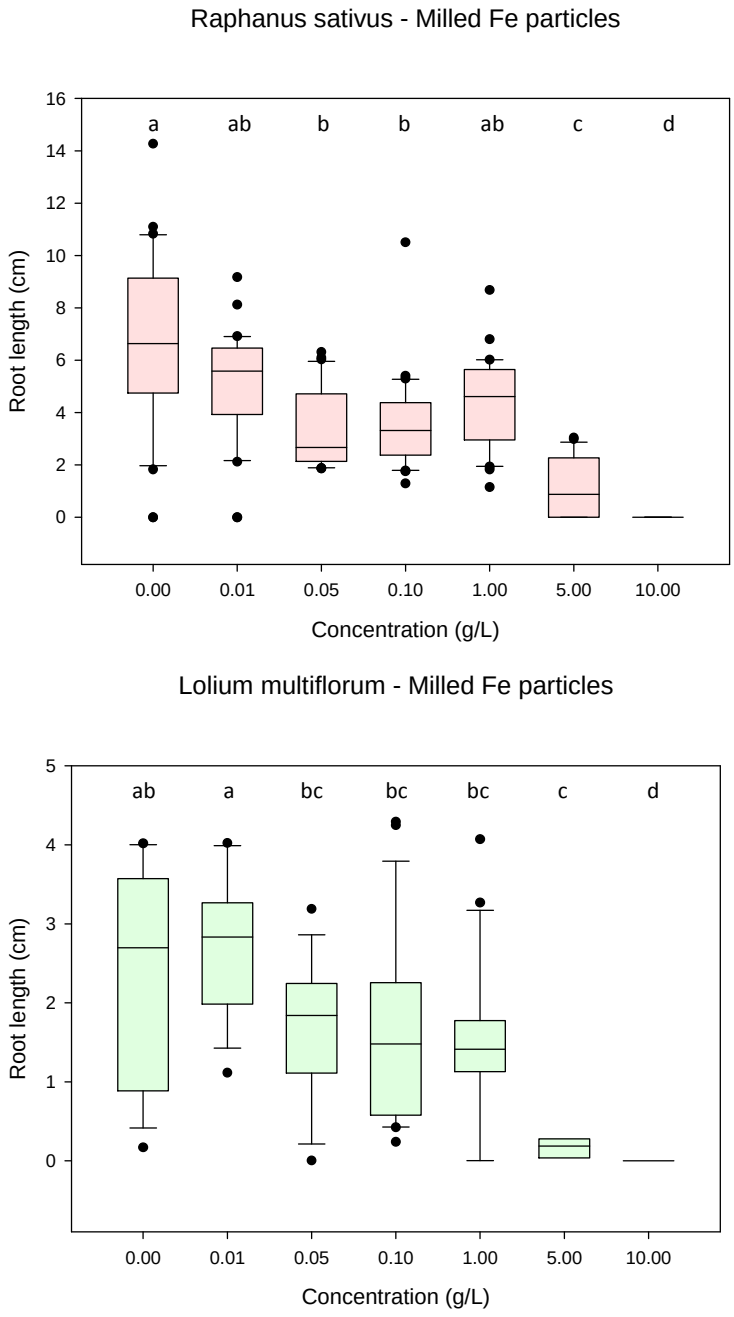


Figure 17 Root length of radish (*Raphanus sativus*) and ryegrass (*Lolium multiflorum*) exposed to various nominal concentrations of milled Fe particles for 6 days. Statistically significant differences are indicated by different letters (ANOVA).

5.1.4 Nano-goethite (HMGU)

- Algal growth test

No signs of toxicity to *P. subcapitata* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with chlorophyll measurements, which rendered the assessment of toxicity unfeasible.

- Algal photosynthesis efficiency test

No adverse effects to *Chlamydomonas* sp. were observed at concentration up to 1000 mg/L, which was the highest concentration of nano-goethite tested (Figure 18).

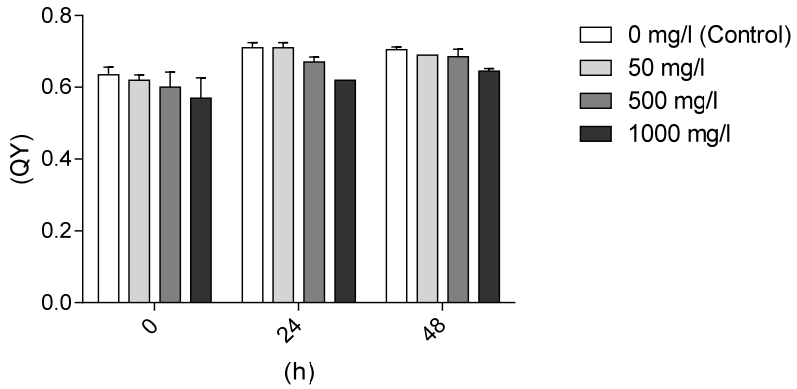


Figure 18 Photosynthesis efficiency (QY) of green alga *Chlamydomonas* sp. exposed to different concentrations of nano-goethite.

- Daphnia immobilization test

No adverse effects to *D. magna* were observed at concentrations up to 1000 mg/L, which was the highest concentration of nano-goethite tested (data not shown).

- Lumbriculus mortality test

No adverse effects to *L. variegatus* were observed at concentrations up to 1000 mg/L, which was the highest concentration of nano-goethite tested (data not shown).

- Bacterial luminescence bioassay

No signs of toxicity to *V. fischeri* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with the measurement of luminescence, which rendered the assessment of toxicity unfeasible.

- E. coli growth test

No adverse effect was observed at concentration up to 1000 mg/L, which was the highest concentration of nano-goethite tested (Figure 12). The pH and ORP of the exposure media are shown in Figure 2.

- E. coli viability test

The percentage of dead cells was 1.7% in the control and 10.8% at the highest nano-goethite concentration tested (1000 mg/L). Lower concentrations of nano-goethite did not impair cell viability (Figures 3 and 13). The pH and ORP of the exposure media are shown in Figure 2.

- Earthworm survival test

For nano-goethite, no adverse effect was observed at Fe concentrations up to 6 g/L (320 µg/cm^2), which was the highest concentration tested (data not shown). All earthworms survived and showed no sign of distress.

- Seed germination and root elongation test

Characterization of the exposure suspensions

The measurements done with nano-goethite include zeta-averaged hydrodynamic diameters measured in the first and sixth (last) day. Parallel to these, zeta potential measurements were also performed on separately stored samples. Additional measurements were performed on samples run in parallel to the exposure tests wich contained double amount of dispersion (10 mL) and 10 seeds and were collected in the final day with the aim of evaluating the effects of organic matter produced by the seeds on the particle stability.

DLS zeta-averaged hydrodynamic diameter measured for samples without seeds in quiescent conditions stored in the DLS cuvettes. In that case, 2 mL of the suspension used for the exposure were kept in dark conditions during the whole duration of the test. Figure 19 shows the photos taken on the final day of the DLS cuvettes with the suspensions tested; even though some material was visible in the sediment, the dispersions remained relatively stable during the duration of the test. Figure 20 shows the results obtained for the zeta-average measured during the duration of the test. An additional measurement was performed for every treatment in the last day where the cuvette was vigorously agitated to resuspend all the material present. In general, the diameters were very stable and slightly lower in the last day indicating that the large fraction of the material had settled.

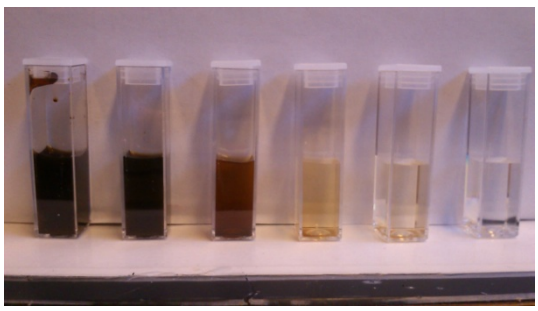


Figure 19 Nano-goethite suspensions used in the plant root elongation test, after 6 days. Concentrations tested were (from left to right) 10, 5, 1, 0.1, 0.05, 0.01 g/L prepared as specified in the protocols.

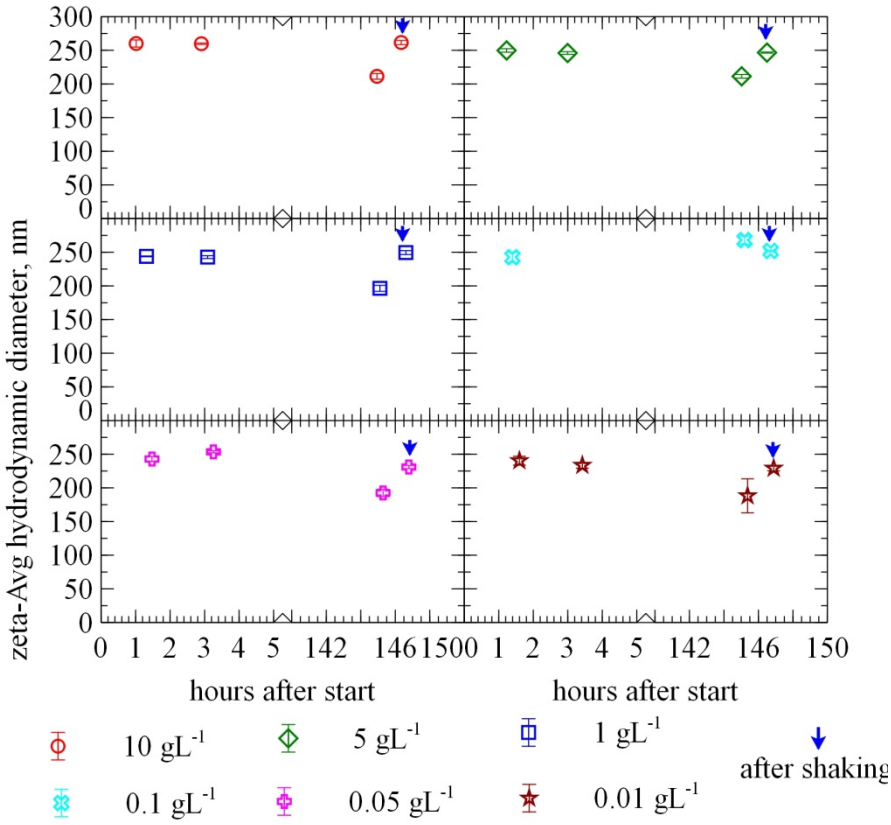


Figure 20 Characterization of aqueous dilutions of nano-goethite used in the plant root elongation test. Zeta-averaged diameters obtained from DLS measurements done in parallel to the tests. Averages of three replicated measurements are presented and error bars represent 1 standard deviation. An additional measurement is presented at the end of the tests after vigorous shaking of the cuvette (arrows).

The measurements of zeta-potential are presented in Figure 21. 6 mL samples were kept in the dark during the duration of the test and approximately 1 mL was extracted with a syringe from the supernatant to measure the zeta-potentials. In general, the zeta-potentials remained negative except for the most concentrated suspension, which showed zeta-potentials around zero at all times.

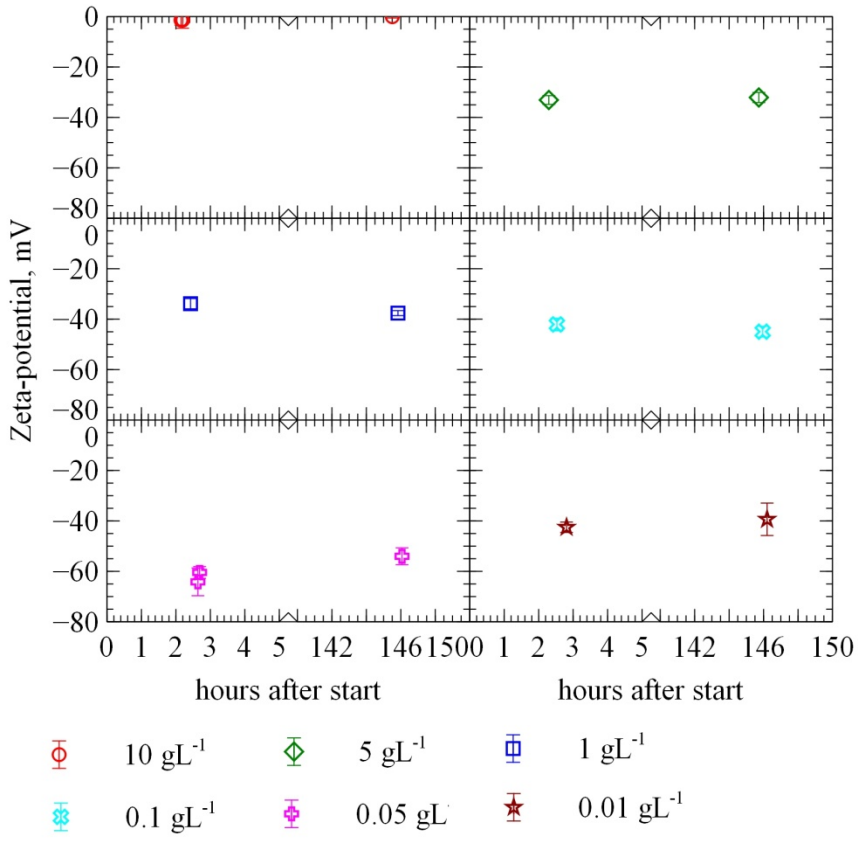


Figure 21 Characterization of aqueous dilutions of nano-goethite used in the plant root elongation test. Zeta-potential values obtained from electrophoretic mobility measurements done in parallel to the tests. Averages of three replicated measurements are presented and error bars represent 1 standard deviation.

An additional experiment was run in parallel with particles and seeds. 10 mL of the particle dispersion was prepared in the same containers as the ecotoxicological experiments were performed but no filter paper was added. The remaining suspension at the end of the experiments was then collected with help of plastic syringes that were used to transfer to zeta-potential cuvettes (Malvern). Particle size and zeta-potentials were measured to identify the possible effects on stability originated by exudates of the seeds during the germination stage. The results are presented in Figure 22.

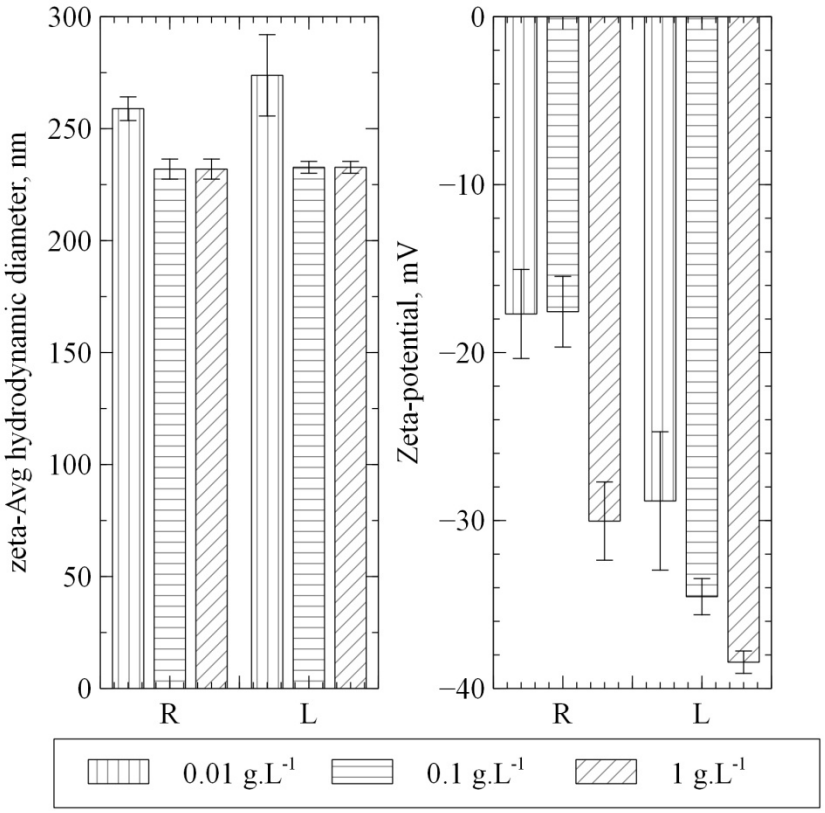


Figure 22 Characterization of aqueous dilutions of nano-goethite used in the plant root elongation test. Hydrodynamic diameter and zeta-potential values obtained from particles together with seeds (R: *Raphanus sativus* and L: *Lolium multiflorum*) done in parallel to the tests for three selected concentrations. Averages of three replicated measurements are presented and error bars represent 1 standard deviation.

The results obtained from these experiments reflect the differences in the particle characteristics derived from exposure to the seeds exudates. The diameters were in the same order of magnitude as those obtained from the suspensions without seeds. Zeta-potential values were smaller in magnitude with the lowest concentrations being more affected than the highest. This can be due to the overall effect of the exudates adsorbed to the particles in the average zeta-potential measured. Measurements of DI water in contact with seeds were not successful, due to lack of signal for the instrument.

The pH of the exposure suspensions (same experimental conditions but without seeds) at the beginning and the end of the test was ranging from 6.7 (control) to 9.0 (from 1 to 10 g/L). The ORP was ranging from 90-100 mV (control and low concentrations) to 60 mV (10 g/L) on day 1, and from 230 mV (control) to 160 mV (10 g/L) on day 6.

Seed germination and root elongation

Nano-goethite did not affect the germination rate of *Raphanus sativus* and *Lolium multiflorum* (highest concentration tested 10 g/L, results not shown). The root length of *R. sativus* was reduced at con-

centrations > 1 g/L. An increase in the root length of *L. multiflorum* was observed from 0 to 1 g/L, followed by a decrease from 1 to 10 g/L (Figure 23).

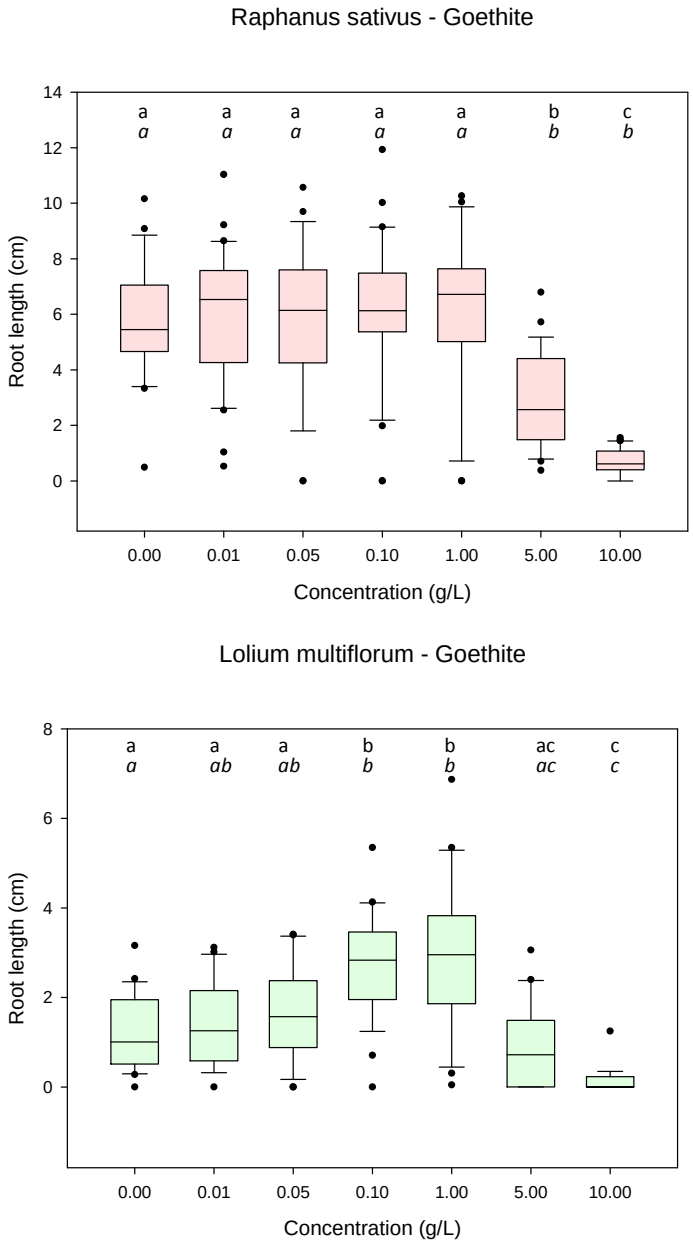


Figure 23 Root length of radish (*Raphanus sativus*) and ryegrass (*Lolium multiflorum*) exposed to various concentrations of nano-goethite for 6 days. Statistically significant differences are indicated by different letters (normal: ANOVA; italics: Kruskal-Wallis ANOVA on ranks)

5.1.5 Fe zeolite (UFZ)

- Algal growth test

No signs of toxicity to *P. subcapitata* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with chlorophyll measurements, which rendered the assessment of toxicity unfeasible.

- Algal photosynthesis efficiency test

No adverse effects to *Chlamydomonas* sp. were observed at concentrations up to 1000 mg/L, which was the highest concentration of Fe zeolite tested (Figure 24).

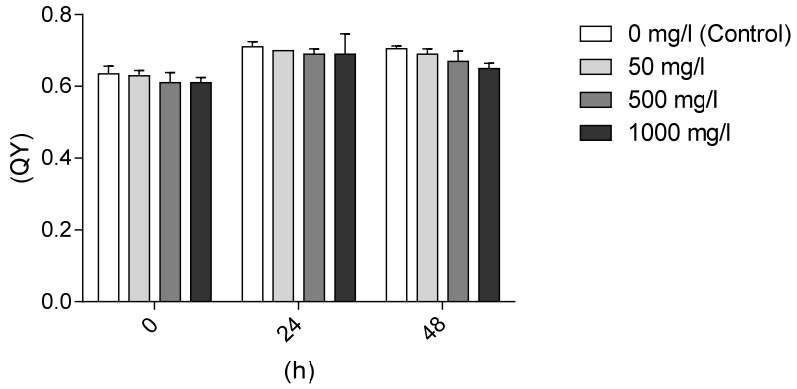


Figure 24 Photosynthesis efficiency (QY) of green alga *Chlamydomonas* sp. exposed to different concentrations of Fe zeolite (Fe-BEA-35).

- Daphnia immobilization test

No adverse effects to *D. magna* were observed at concentrations up to 1000 mg/L, which was the highest concentration of Fe zeolite tested (data not shown).

- Lumbriculus mortality test

No adverse effects to *L. variegatus* were observed at concentrations up to 1000 mg/L, which was the highest concentration of Fe zeolite tested (data not shown).

- Bacterial luminescence bioassay

No signs of toxicity to *V. fischeri* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with the measurement of luminescence, which rendered the assessment of toxicity unfeasible.

- E. coli growth test

The EC_{10} was 382 mg/L Fe zeolite, the highest concentration tested was 1000 mg/L (Figure 12). The pH and ORP of the exposure media are shown in Figure 2.

- E. coli viability test

Fe zeolite did not impair the viability of *E. coli* after 24h exposure to concentrations up to 1000 mg/L. The percentage of dead cells at 1000 mg/L (1.0%) was similar to the control (1.7%) (Figures 3 and 13). The pH and ORP of the exposure media are shown in Figure 2.

- Earthworm survival test

No adverse effect was observed at Fe zeolite concentrations up to 25 g/L (700 µg/cm^2), which was the highest concentration tested (data not shown). All earthworms survived and showed no sign of distress.

- Seed germination and root elongation test

Characterization of the exposure suspensions

The measurements done with Fe zeolite include zeta-averaged hydrodynamic diameters measured in the first, second and sixth (last) day. Parallel to these, zeta potential measurements were also performed on separately stored samples. On the third day selected samples without seeds were analyzed with NTA to evaluate the presence of small (<~600 nm hydrodynamic diameter) particles. Additional measurements of zeta-average diameter and zeta-potentials were performed on samples collected in the final day from the containers containing the seeds. In some cases, the presence of very large particulate material was detected but it was not possible to elucidate if it belonged to the original particles or if it was degradation products from the filter paper. Zeta-potentials in these samples were always negative and around -20 mV (data not presented).

Zeta-averaged hydrodynamic diameter

DLS zeta-averaged hydrodynamic diameter measured for samples without seeds in quiescent conditions stored in the DLS cuvettes. In this case, 2 mL of the suspension used for the exposure were kept in dark conditions during the whole duration of the test. Figure 25 shows the photos of the DLS cuvettes with the suspensions tested. Figure 26 shows the results obtained for the zeta-average measured during the duration of the test. An additional measurement was performed for every treatment in the last day where the cuvette was vigorously agitated to resuspend all the material present. In general, the diameter was much larger after shaking the cuvettes indicating that a large part of the material was large enough to settle and influenced significantly the DLS measurements. The supernatant in the last day contained sufficient particles to generate reliable signals from the DLS instrument and have average diameters between 200 and 400 nm for the lowest concentrations (0.01 to 1 g/L) and 500 and 1000 nm for 5 and 10 g/L, respectively.

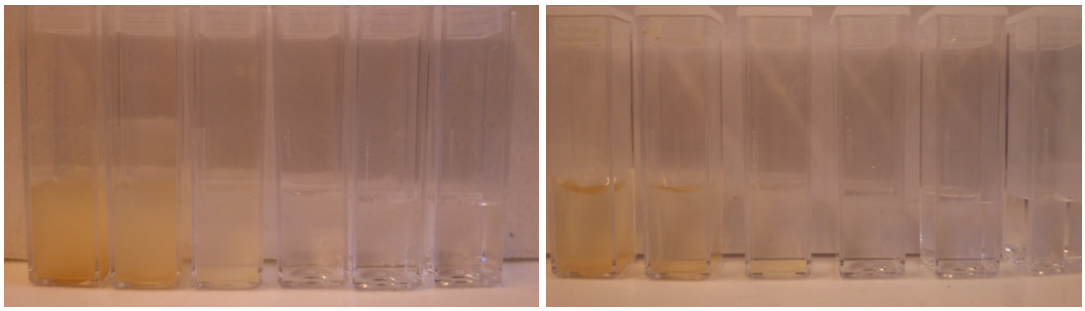


Figure 25 Photos taken on the first (left) and last day (right) of the zeolite Fe-BEA-35 suspensions used in the root elongation test. Concentrations tested were (from left to right) 10, 5, 1, 0.1, 0.05, 0.01 g/L prepared as specified in the protocol sent by UFZ.

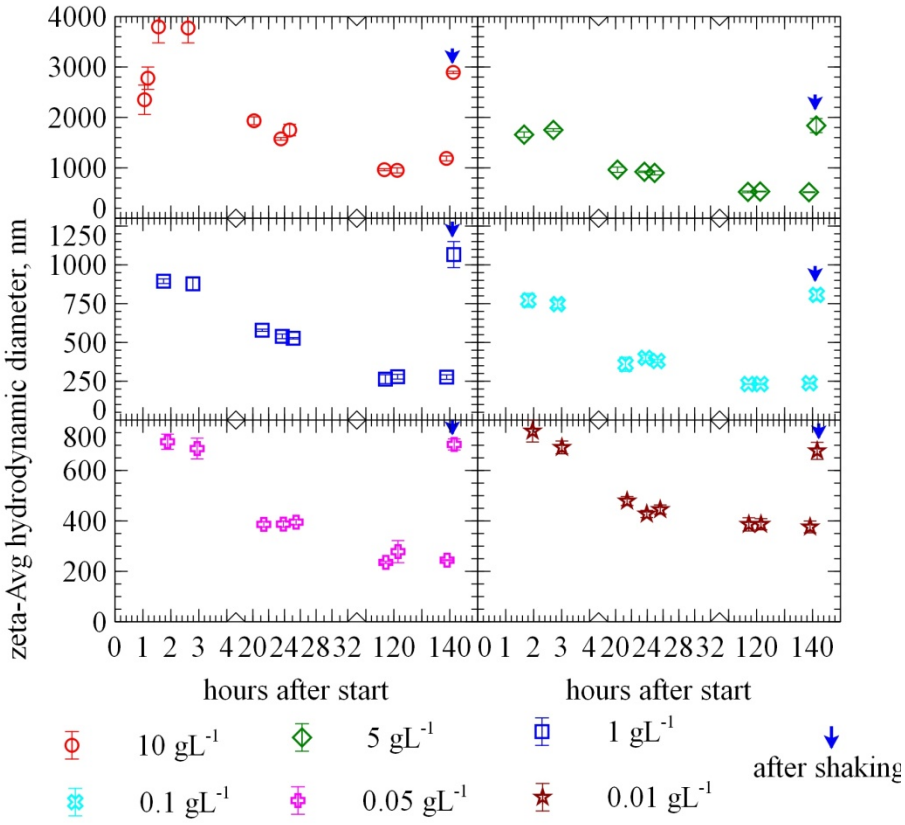


Figure 26 Zeta-averaged diameters of zeolite Fe-BEA-35 obtained from DLS measurements done in parallel to the root elongation test. Averages of three replicated measurements are presented and error bars represent 1 standard deviation. An additional measurement is presented at the end of the tests after vigorous shaking of the cuvette (arrows).

Zeta-potential

The measurements of zeta-potential are presented in Figure 27. 6 mL samples were kept in dark during the duration of the test and approximately 1 mL was extracted with a syringe from the supernatant to measure the zeta-potentials. In the last day and after measuring the supernatant, the samples were vigorously mixed and zeta-potential were also measured. In general, the zeta-potentials of the mixed dispersion were more negative than the measured in the supernatant. All the suspensions presented relatively low negative zeta-potentials indicating stability with respect to aggregation.

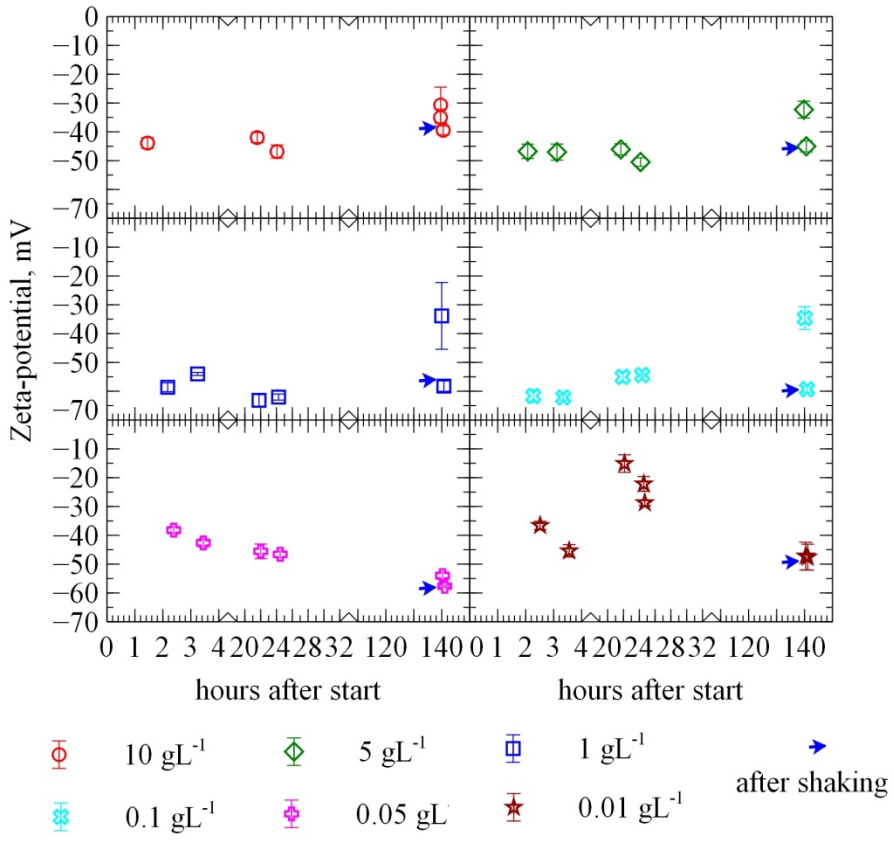


Figure 27 Characterization of aqueous suspensions of zeolite Fe-BEA-35 used in the root elongation test. Zeta-potential values obtained from electrophoretic mobility measurements done in parallel to the tests. Averages of three replicated measurements are presented and error bars represent 1 standard deviation. An additional measurement is presented at the end of the tests after vigorous shaking of the container (arrows).

NTA

Additional measurements were performed in NTA with the three lowest concentrations (Figure 28). These concentrations were in the range of concentration (number-based) that NTA can handle. The results confirm the presence of material between 200 and 400 nm in these samples. The presence of large particles was not observed when performing the measurements even if they were not avoided intentionally; this suggests that the number of such big particles is not significant in number compared to the small ones.

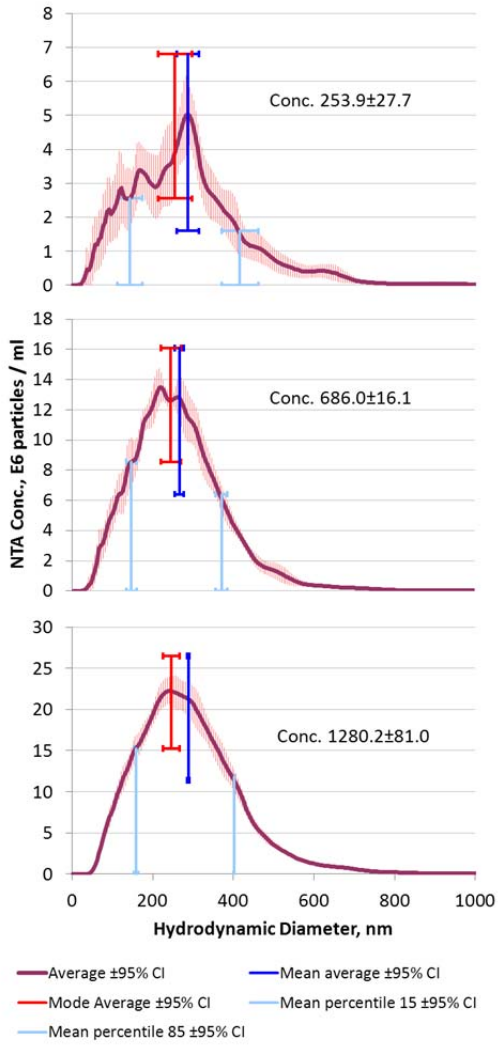


Figure 28 Particle-size distribution (PSD) of the aqueous suspensions of zeolite Fe-BEA-35 with concentration 0.01 g/L (up), 0.05 g/L (middle) and 0.1 g/L (low). Complete lines correspond to average values of seven videos of 60 s each. Error bars represent the 95% confidence interval (CI) among the videos. Mean values, mode values, 15th and 85th percentiles were calculated for each video and are presented in the PSD as average values of the seven videos with the respective 95% CI. Inserts are total number concentrations presented for each sample with the corresponding 95% CI.

Seed germination and root elongation

Fe zeolite did not affect the germination rate of *Raphanus sativus* and *Lolium multiflorum* (highest concentration tested 10 g/L, results not shown). The root length of *R. sativus* and *L. multiflorum* was was reduced at concentrations > 1 g/L and > 5 g/L, respectively (Figure 29).

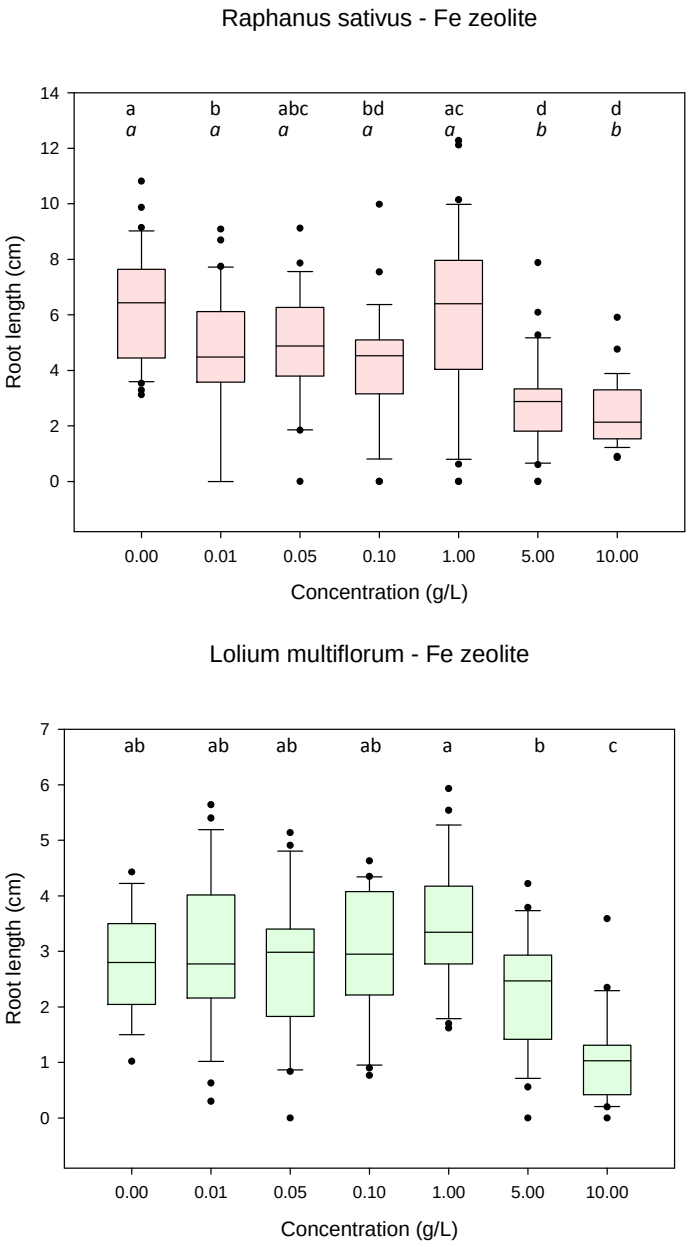


Figure 29 Root length of radish (*Raphanus sativus*) and ryegrass (*Lolium multiflorum*) exposed to various concentrations of Fe zeolite for 6 days. Statistically significant differences are indicated by different letters (normal: ANOVA; italics: Kruskal-Wallis ANOVA on ranks).

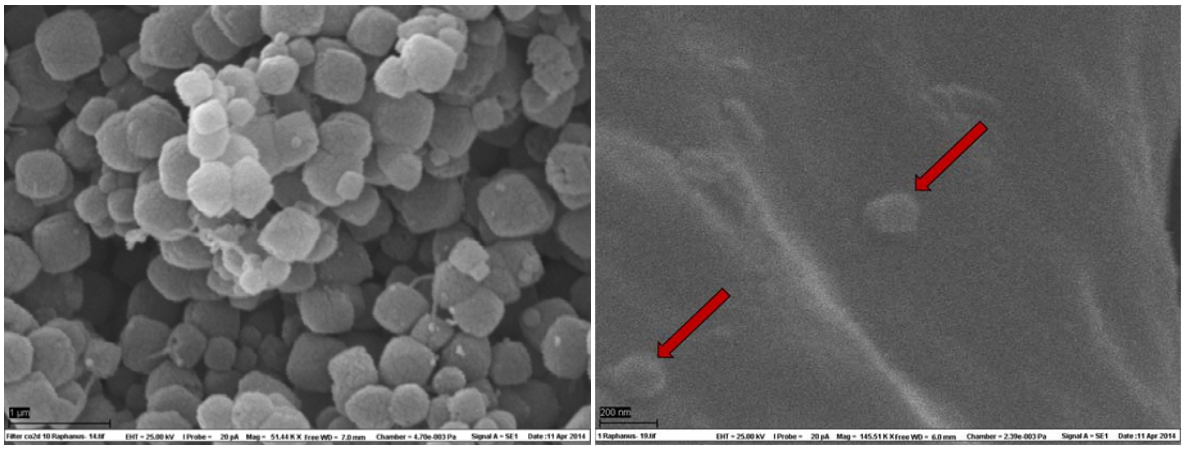


Figure 30 Scanning electron micrographs of (left picture) Fe zeolite on filter paper at the end of the germination test; (right picture) individual Fe zeolite particles strongly attached to a radish root (exposure concentration 1 g/L).

5.1.6 Carbo-Iron and associated (UFZ)

- Algal growth test

No signs of toxicity to *P. subcapitata* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with chlorophyll measurements, which rendered the assessment of toxicity unfeasible.

- Algal photosynthesis efficiency test

No adverse effects to *Chlamydomonas* sp. were observed at concentrations up to 100 mg/L for reactive Carbo-Iron, aged Carbo-Iron, activated carbon and up to 200 mg/L for CMC (Figure 31, results for CMC not shown). The tested concentrations were the highest concentrations we were able to test, due to nanoparticle interference with the detection method.

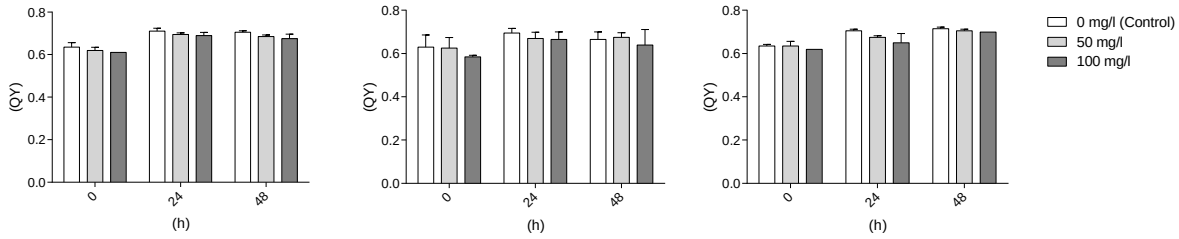


Figure 31 Photosynthesis efficiency (QY) of green alga *Chlamydomonas* sp. exposed to different concentrations of reactive Carbo-Iron (left), aged Carbo-Iron (middle), activated carbon (right).

- Daphnia immobilization test

No adverse effects to *D. magna* were observed for Carbo-Iron at concentrations up to 100 mg/L (data not shown). Higher concentrations made difficult the observation of daphnids, due to the darkness of the suspension. The activated carbon affected the mobility of the daphnia at concentrations above 50 mg/L. Aged Carbo-Iron and CMC have not been assessed in this test.

- Lumbriculus mortality test

No adverse effects to *L. variegatus* were observed at concentrations up to 1000 mg/L, which was the highest concentration of reactive Carbo-Iron tested (data not shown).

- Bacterial luminescence bioassay

No signs of toxicity to *V. fischeri* were observed at concentrations up to 100 mg/L (data not shown). At higher concentrations, the particles interfered with the measurement of luminescence, which rendered the assessment of toxicity unfeasible.

- E. coli growth test

No adverse effects to *E. coli* were observed at concentrations up to 1000 mg/L for reactive Carbo-Iron (the highest concentration tested, Figure 12). The EC_{10} was 500 mg/L for aged Carbo-Iron and 385 mg/L for activated carbon. The pH and ORP of the exposure media are shown in Figure 2.

- E. coli viability test

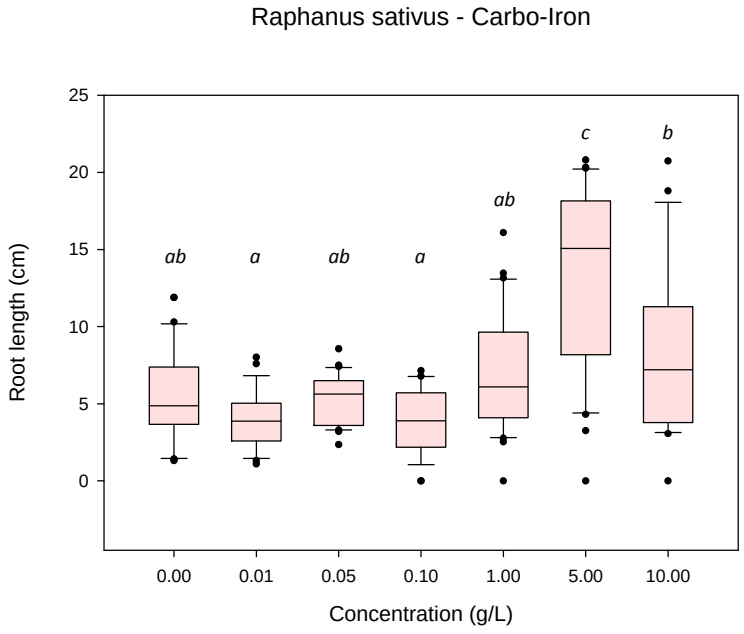
The percentage of dead *E. coli* cells at the highest tested concentration 1000 mg/L was 12.0% for activated carbon, 5.0% for reactive Carbo-Iron (Figures 3 and 13), 14.2% for aged Carbo-Iron, and 1.7% in the control. The pH and ORP of the exposure media are shown in Figure 2.

- Earthworm survival test

No adverse effect was observed at concentrations up to 10 g/L (280 µg/cm^2), which was the highest concentration tested for reactive Carbo-Iron, aged Carbo-Iron, activated carbon, and CMC (data not shown). All earthworms survived and showed no sign of distress.

- Seed germination and root elongation test

No adverse effect on the root elongation of *Raphanus sativus* and *Lolium multiflorum* was observed in presence of reactive Carbo-Iron, aged Carbo-Iron and activated carbon. Increasing concentrations of reactive Carbo-Iron enhanced root elongation of these plant species (Figure 32).



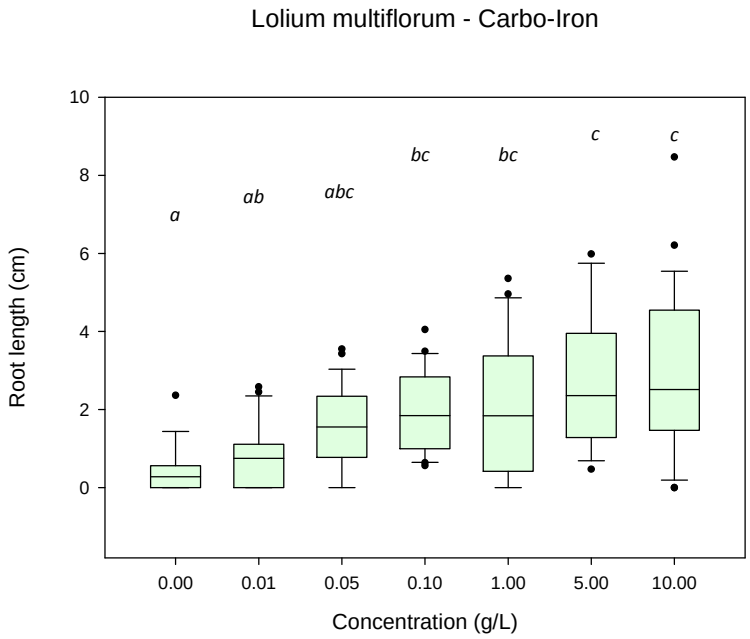


Figure 32 Root length of radish (*Raphanus sativus*) and ryegrass (*Lolium multiflorum*) exposed to various concentrations of reactive Carbo-Iron for 6 days. Statistically significant differences are indicated by different letters (Kruskal-Wallis ANOVA on ranks).

5.1.7 Bio-nanomagnetite (UMAN)

- Earthworm survival

No adverse effect was observed at Fe concentrations up to 7 g/L (420 µg/cm^2), 11 g/L (600 µg/cm^2), and 5 g/L (290 µg/cm^2), for biomagnetite, palladised biomagnetite, and commercial magnetite, respectively, which were the highest concentrations tested (data not shown). All earthworms survived and showed no sign of distress.

5.2 Matrix effects on Ecotox

Except the milled Fe particles KKM 03-06, none of the nanoparticles were toxic to organisms in simple exposure media, expected to provide high bioavailability/exposure to organisms. Therefore, we decided to limit toxicity testing in presence of humic acids to the milled Fe particles. In addition, nZVI particles (with 1% CMC) made by borohydride reduction of $FeCl_3$ were also tested, as a particle positive control (Lien and Zhang, 2001). The hypothesis was that humic acids would reduce the toxicity of milled Fe particles to organisms by reducing their bioavailability.

Milled Fe particles are composed of zero-valent Fe, and have a limited shelf life. Therefore, the producer UVR-FIA recommended that we used a newer batch of particles, and sent us freshly prepared particles that were tested within one week after receipt. The results presented below have been obtained from tests conducted on milled Fe particles KKM 14.

5.2.1 Bacterial growth test

Characterization of the exposure media

The zeta potential of milled Fe particles KKM 14 and positive control nZVI are presented in Figure 33; the average hydrodynamic diameter (by Dynamic Light Scattering, DLS) in Figure 34, and the average particles size (by Differential Centrifugal Sedimentation, DCS) in Figure 35.

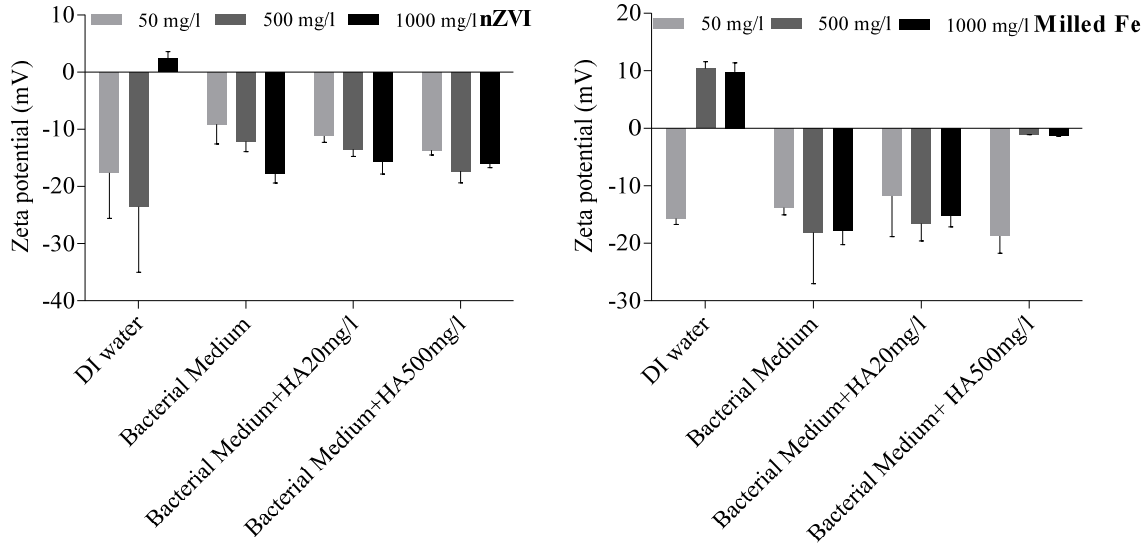


Figure 33 Zeta potential (mV) of positive control nZVI (left) and milled Fe particles (right) suspended in DI water, bacterial medium and bacterial medium with humic acids (20 and 500 mg/L). Each measurement was done in triplicated runs.

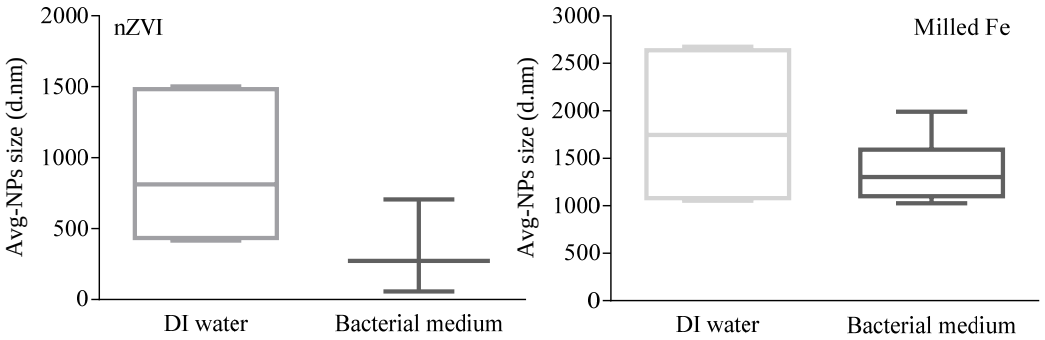


Figure 34 Average hydrodynamic diameter (determined by DLS) of positive control nZVI (left) and milled Fe particles (right) suspended in DI water and bacterial medium. Each measurement was done in triplicated runs.

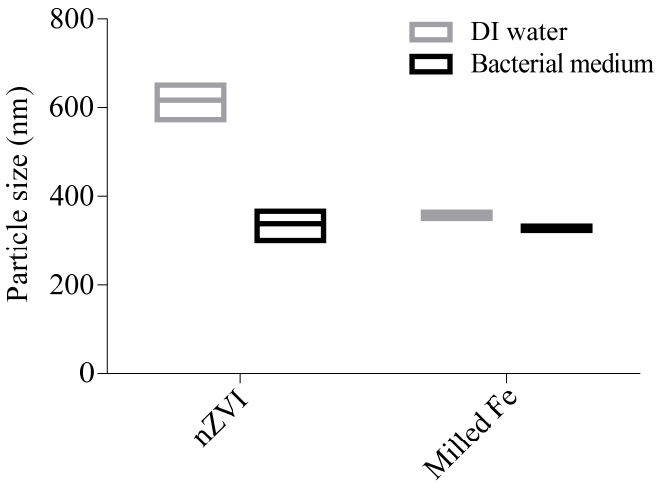


Figure 35 Average particle size (determined by DCS) of positive control nZVI (left) and milled Fe particles (right) suspended in DI water and bacterial medium. Each measurement was done in triplicated runs.

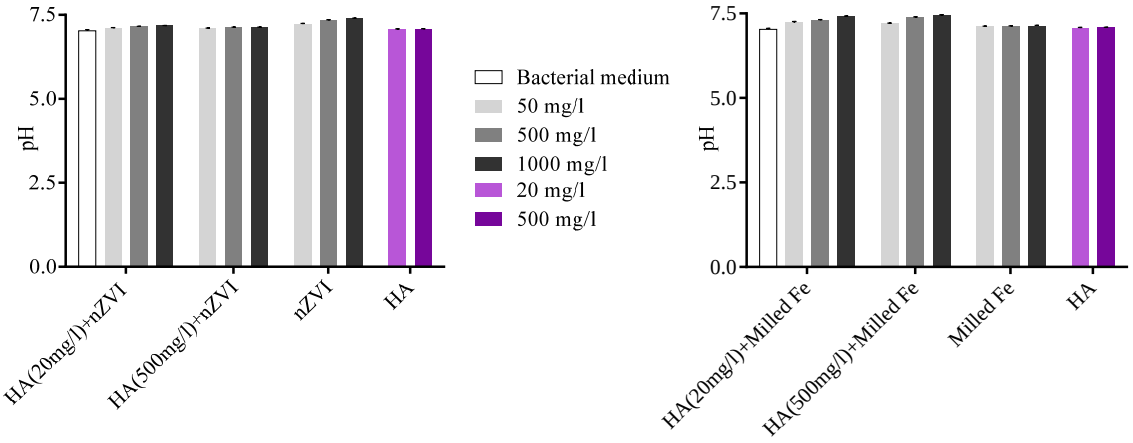


Figure 36 pH values of positive control nZVI (left) and milled Fe particles (right) suspended in bacterial growth medium, with and without humic acids (HA).

Bacterial growth test results

The growth rate of anaerobic bacteria *C. perfringens* exposed to milled Fe particles or positive control nZVI in presence/absence of humic acid was followed over a 24h period. No change in toxicity was observed in the presence of humic acid (Fig. 37). The positive control nZVI was more toxic to *C. perfringens* than the milled Fe particles, as expected.

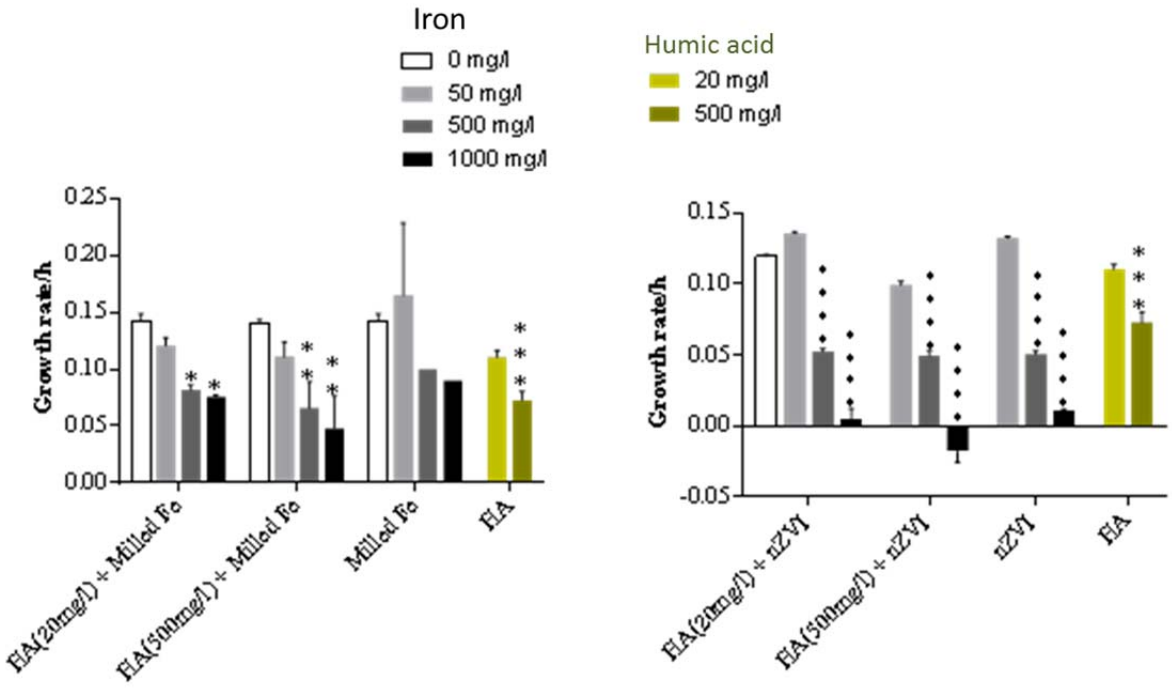


Figure 37 Growth rate of *C. perfringens* after 24h exposure to milled Fe particles (left) and positive control nZVI (right) in the presence/absence of humic acid (HA).

5.2.2 Algal assimilation test

Characterization of the exposure media

Determination of particle size distribution, hydrodynamic diameter and zeta potential of the milled Fe particles and the positive control nZVI suspended in algal media (ISO 8692, 2012) with and without 20 mg/L humic acid (Suwannee river NOM) was attempted by DLS. It revealed that all particle suspensions had a very broad size distribution with polydispersity indexes around 1, which undermines the use of DLS measurements.

Algal assimilation test results

In general, no change in toxicity was observed with or without the addition of humic acid, as the tested particles did not significantly inhibit the biomass assimilation of ^{14}C compared to controls (data not shown). On the contrary, most tested concentrations of both particles with and without humic acid showed higher rates of assimilation than controls, as the abundance of iron can be a stimulating factor for photosynthesis.

6 Conclusions

The results of ecotoxicity tests using the particles developed during the first two years of the project did not show any toxicity to organisms. The only exception was the milled Fe particles KKM 03-06. However, bacterial and agal tests performed on another (newer) batch of milled Fe particles (batch KKM 14) did not show the same high toxicity as found with batch KKM 03-06. The producer UVR-FIA told us that these batches were supposed to be similar, since they were produced following the same protocol. The reasons for the difference in toxicity between the two batches are therefore still unknown.

The low toxicities found in the standard organisms do not lead to any hazard classification according to EU regulation for any of the tested particles and the results indicate that the particles, except the milled ZVI particles, can be considered non-toxic.

In the absence of intrinsic toxicity of most of the particles, the second part of the deliverable was limited to tests on milled Fe particles. The presence of humic acid did not change the outcome of the ecotox tests, contrary to what was expected.

7 List of References

ISO (2007). ISO 11348-3. Water quality - determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (luminescent bacteria test), Geneva, Switzerland.

ISO (2012). ISO 8692. Water quality - Fresh water algal growth inhibition test with unicellular green algae, Geneva, Switzerland.

Water quality -- Fresh water algal growth inhibition test with unicellular green algae

Lien HL and Zhang WX (2001). Nanoscale iron particles for complete reduction of chlorinated ethenes. Colloid and Surfaces A, 191:97-105.

Mayer P, Cuhel R, Nyholm N (1997). A simple in vitro fluorescence method for biomass measurements in algal growth inhibition. Water Research, 31(10):2525-2531

OECD (1984). OECD guideline 207 for testing of chemicals. Earthworm, acute toxicity tests. Paris, France.

OECD (2004). OECD guideline 202 for testing of chemicals. Daphnia sp., Acute Immobilisation. Paris, France.

OECD (2006). OECD guideline 208 for testing of chemicals. Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test. Paris, France.

OECD (2008). OECD guideline 315 for testing of chemicals. Bioaccumulation in Sediment-dwelling Benthic Oligochaetes. Paris, France.

OECD (2011). OECD guideline 201 for testing of chemicals. Freshwater Alga and Cyanobacteria, Growth Inhibition. Paris, France.

Raghupathi KR, Koodali RT, Manna AC. (2011). Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. Langmuir, 27(7):4020-8.

Sørensen SN and Baun A. (2014). Controlling silver nanoparticle exposure in algal toxicity testing - A matter of timing. Nanotoxicology, 5390:1-9.

USEPA (1996). OPPTS 850.4200 Seed Germination/Root Elongation Toxicity Test. USA.