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Ecotoxicity testing and environmental risk assessment of iron nanomaterials for sub-surface remediation – Recommendations from the FP7 project NanoRem

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Abstract

Nanoremediation with iron (Fe) nanomaterials opens new doors for treating contaminated soil and groundwater, but is also accompanied by new potential risks as large quantities of engineered nanomaterials are introduced into the environment. In this study, we have assessed the ecotoxicity of four engineered Fe nanomaterials, specifically, Nano-Goethite, Trap-Ox Fe-zeolites, Carbo-Iron[®] and FerMEG12, developed within the European FP7

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project NanoRem for sub-surface remediation towards a test battery consisting of eight ecotoxicity tests on bacteria (*V. fischeri*, *E. coli*), algae (*P. subcapitata*, *Chlamydomonas* sp.), crustaceans (*D. magna*), worms (*E. fetida*, *L. variegatus*) and plants (*R. sativus*, *L. multiflorum*). The tested materials are commercially available and include Fe oxide and nanoscale zero valent iron (nZVI), but also hybrid products with Fe loaded into a matrix. All but one material, a ball milled nZVI (FerMEG12), showed no toxicity in the test battery when tested in concentrations up to 100 mg/L, which is the cutoff for hazard labeling in chemicals regulation in Europe. However it should be noted that Fe nanomaterials proved challenging to test adequately due to their turbidity, aggregation and sedimentation behavior in aqueous media. This paper provides a number of recommendations concerning future testing of Fe nanomaterials and discusses environmental risk assessment considerations related to these.

Keywords: Nanoremediation, Iron nanomaterials, Ecotoxicology, nZVI, Environmental Risk Assessment, NanoRem

1 Introduction

Innovation in nanotechnology introduces new treatment options for environmental remediation of organic compounds (notably chlorinated solvents) and heavy metals in soil and groundwater (Karn et al., 2009;

Mueller et al., 2012). Especially iron (Fe) based nanomaterials have shown a potential for remediation due to a larger specific surface area and corresponding increased reactivity compared to micro-scale and larger Fe materials traditionally used for remediation of contaminated sites (Wang and Zhang, 1997). Nanoscale zerovalent iron (nZVI) has received most of the attention as it is highly reactive compared to the bulk ZVI used in permeable reactive barriers (Henderson and Demond, 2007). Remediation with nZVI has been claimed to represent a faster, cheaper and a potentially more effective treatment option than current *ex situ* and *in situ* methods (Yan et al., 2013). However, nanomaterials engineered to remediate polluted soil and groundwater may constitute a risk to the environment as they are injected into the subsurface in large quantities (Grieger et al., 2010). As such this could represent a worst case scenario when considering possible negative environmental effects of manufactured nanomaterials. Nanoremediation in general seems associated with high uncertainty both in relation to its potential environmental risks, but also towards its field scale efficacy (Grieger et al., 2015). Uncertainty with regards to the potential environmental impacts of Fe nanomaterials hampers their use and has partly been the reason for the limited implementation of Fe nanomaterials in remediation (Bardos et al., 2014), although no major environmental impacts have been reported in the first decade of field deployments with Fe nanomaterials (Mueller et al., 2012).

Several publications in recent years have evaluated the ecotoxicity of Fe nanomaterials, particular nZVI materials, with NANOFER STAR, NANOFER 25 and 25s being the most common commercially available particles. In these publications the focus has been on aquatic and terrestrial ecotoxicity (Keller et al., 2012; Marsalek et al., 2012; Saccà et al., 2014; El-Temsah et al., 2016) and in general effect concentrations as low as 0.5 mg/L to above 2.5 g/L have been reported, demonstrating considerable variation in ecological response to Fe nanomaterials.

A range of Fe nanomaterials has been developed in the European FP7 project NanoRem (Taking Nanotechnological Remediation Processes from Lab Scale to End User Applications for the Restoration of a Clean Environment, for more information see nanorem.eu) in order to extend the spectrum of treatable soil and groundwater contaminants from halogenated organics to non-halogenated substances and non-reducible metals. Contrary to the three materials mentioned above, the potential ecotoxicity of the materials developed in NanoRem have not previously been tested. These materials are currently available on the international market (see Table 1). Common for all materials is that if they are to be used in field-scale remediation, their production volume will easily reach 1 metric ton per year (Mueller et al., 2012). In this case, they will have to be registered under the European chemical legislation REACH, which will be accompanied with data requirements on ecotoxicity. The data generated will feed into the general

hazard identification of the nanomaterials and form the basis for a generic risk assessment (i.e. a hazard classification according to the classification, labeling and packaging (CLP) regulation). It is important to emphasize that this risk assessment does not directly relate to the safety of injecting said material into an aquifer or a contaminated soil. Such a task is done in a site-specific risk assessment, which is outside the scope of this study.

Test organisms and endpoints in the ecotoxicology test battery were chosen to include representatives for both terrestrial and aquatic environments as Fe nanomaterials may spread, in worst case scenarios, to both terrestrial and aquatic habitats (Grieger et al., 2010). The ecotoxicity tests were also selected to include standardized tests to ensure general regulatory acceptance of test results as well as non-standardized tests to broaden the test basis with respect to modes of exposure and modes of action, and to enhance the likelihood of seeing biological responses within the range of particles and concentrations tested. The aim of the paper is to provide ecotoxicity data for four newly developed Fe nanomaterials and the paper also highlights current challenges in doing adequate hazard identification and environmental risk assessment of Fe nanomaterials. Finally, recommendations for future ecotoxicity testing of Fe nanomaterials are provided.

2 Materials and Methods

2.1 Nanomaterials

Samples of Fe nanomaterials for ecotoxicity testing were obtained directly from the manufacturers within the NanoRem project. A full list and characterization of the nanomaterials is seen in Table 1.

2.1.1 Dispersion of nanomaterials for toxicity testing

Dispersions of powder Fe nanomaterials were made according to the description provided by the manufactures. Due to testing constraints (e.g. infeasibility to degas exposure media) for the aquatic standard tests (on *V. fischeri*, *P. subcapitata* and *D. magna*), all nanomaterial powders were dispersed as described for magnetite.

Carbo-Iron[®] For 100 mL of a stock suspension at 10 g/L, 20 mL of a 10 g/L carboxymethyl cellulose (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 N₂ flow, and mixed for 10 min with a high-shear mixer. Dilution series were prepared under regular aerobic conditions and used right away.

Trap-Ox Fe-zeolites A stock suspension with a zeolite concentration of 25 g/L was prepared by dissolving 2.5 g CMC in 50 mL deionized water by heating the mixture to 70°C with stirring for an hour. Then, 2.5 g Fe-zeolite

in 50 mL deionized water was sonicated for 15 min and the CMC solution and zeolite suspension were mixed and sonicated for additional 15 min.

Magnetite Magnetite, received as powder, was suspended in deionized water and mixed for 10 minutes with a high-shear mixer. Subsequent dilutions series in exposure media were prepared and used right away.

Suspensions Nano-Goethite was provided as a stable suspension and was diluted directly from the sample into the exposure media. However, the FerMEG12 were additionally sonicated for 15 minutes due to sedimentation.

2.2 Characterization of stock suspensions

Dynamic Light Scattering (DLS) measurements of aqueous suspensions (deionized water) from 10 mg/L to 10 g/L test material 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 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.

149 Nanoparticle Tracking Analysis (NTA) measurements of the hydrodynamic
150 diameter of individual particles suspended in deionized water at a
151 concentration of 10 mg/L to 10 g/L were done on a Nanosight LM10
152 (NanoSight Ltd, Amesbury, UK).

Table 1 Characterization of the pristine Fe nanomaterials. Magnetite is not used for remediation in NanoRem but was chosen as a control in this study. The listed information is obtained from the manufacturer.

Name	Description	Development Status	Mode of remediation	Form	Chemical composition	Average primary particle size (nm)	Specific surface area (m ² /g)
FerMEG12	Zero-valent Fe mechanically ground through ball milling	Field tested and commercially available	Reduction	Suspension	15-30% Fe 70-85 % monoethylene glycol	-	12-18
Carbo-Iron [®]	Composite of activated carbon and zero-valent Fe	Field tested and commercially available	Adsorption + Reduction	Powder	30.3 % Fe _{tot} 20.5 % Fe ⁰ 13.1 % Fe ₃ O ₄ 55±1% C _{tot}	13440 ±20	594
Magnetite	Fe oxides (Fe ₃ O ₄)	Precursor for NANOFER STAR	-	Powder	Fe ₃ O ₄	-	-
Nano-Goethite	Fe oxides stabilized with humic acids	Field tested and commercially available	Adsorption + Oxidation	Suspension	'pure' FeOOH with organic coating	220±20	140
Trap-Ox Fe-zeolites	Nanoporous aluminosilicate loaded with Fe(III)	Premarket	Adsorption + Oxidation	Powder	4 % Al 92 % Si 3 % Fe	1000	600

The light source was a solid-state, single-mode laser diode (radiation output max power $<50\mu\text{W}$, 635nm continuous wave, max power $< 35\text{mW}$). 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. The solution oxidation-reduction potential and pH were measured in all exposure suspensions at the beginning and the end of the tests. Total Fe concentration in stock suspensions was measured by ICP-OES (Perkin Elmer, Optima 5300 DV) following microwave assisted digestion under acidic conditions (3.7 % HCl).

2.3 Ecotoxicological test battery

A test battery of eight tests (see Table 2) was used to assess and rank the nanomaterials listed in Table 1. Dilutions series were made from stock suspensions and tested in concentrations up to 1 g/L. For some tests, higher concentrations were assessed, including the root elongation test with radish *Raphanus sativus*, ryegrass *Lolium multiflorum* (up to 10 g/L) and the earthworm mortality test with *Eisenia fetida* (up to 25 g/L). Full tests protocols are enclosed in the supplementary information.

Table 2 Organisms and testing endpoints of the eight ecotoxicity tests in the test battery.

Organism	Species	Duration	Endpoint	Reference
Bacteria	<i>Vibrio fischeri</i>	15 min	Decrease in bioluminescence	ISO11348-3
Bacteria	<i>Escherichia coli</i>	6h/24 h	Growth/Cell viability	-
Algae	<i>Pseudokirchneriella subcapitata</i>	48 h	Growth rate inhibition	OECD 201
Algae	<i>Chlamydomonas</i> sp.	48 h	Photosynthesis efficiency	-
Crustacean	<i>Daphnia magna</i>	48 h	Immobilization	OECD 202
Earthworm	<i>Eisenia fetida</i>	48 h	Mortality	OECD 207
Oligochaete	<i>Lumbriculus variegatus</i>	96 h	Mortality	OECD 225 ^a
Plant	<i>Raphanus sativus</i> , <i>Lolium multiflorum</i>	6 d	Root elongation	OECD 208

^a modified to short term water-phase exposure

3 Results

3.1 Characterization

Table 3 provides an overview of the characterization of the Fe nanomaterials in deionized water. Differences in size distribution were observed, with NTA generally finding a lower average size than DLS. Based on zeta potential measurements, Nano-Goethite and Trap-Ox Fe-zeolites showed higher aqueous stability than FerMEG12 and Carbo-Iron[®], however sedimentation of the Trap-Ox Fe-zeolites was also observed. In the more complex aquatic test media, DLS measurements proved difficult due to particle sedimentation of all tested materials. This violates the principle behind DLS for size

distribution measurements as the particles are affected by gravitational movement and not just Brownian movement and no reliable estimation of size distribution could be made. Characterization with DLS also revealed that all particle suspensions had a very broad size distribution with polydispersity indexes around 1, which also undermines the use of DLS measurements to characterize the suspensions.

3.2 Ecotoxicity

Almost all of the tests conducted showed no toxicity of the tested Fe nanomaterials at concentrations up to 100 mg/L, which is the cutoff value for hazard labeling in the EU. Only FerMEG12 gave rise to toxicity at concentrations below 100 mg/L. Effects were seen in the 6 h growth inhibition test with *E. coli* (Figure 1), the 6 d root elongation test with *R. sativus* (Figure 2) and 96 h mortality test with *L. variegatus* (Figure 3).

The growth rate (h^{-1}) of Gram-negative *E. coli* was not significantly affected in the presence of Carbo-Iron[®], Nano-Goethite and Trap-Ox Fe-zeolites at any of the tested concentrations (Figure 1). A significant effect on *E. coli* growth rate was observed for FerMEG12, from concentrations as low as 50 mg/L ($P < 0.001$), and for magnetite at the highest concentration tested (1000 mg/L, $P < 0.05$).

Table 3 Characterization of the four tested nanomaterials dispersed in deionized water at 100 mg/L. Samples were characterized 2 and 144 h after dispersion and analyzed by DLS for hydrodynamic diameter and zeta-potential. NTA analysis was performed in samples 2 h after dispersion.

Nanomaterial	DLS Hydrodynamic diameter (z-average; nm)		Zeta-potential (mV)		NTA Average size (mode average; nm)
	2 h	144 h	2 h	144 h	
FerMEG12	480	720	12	1.5	210
Carbo-Iron [®]	1300	500	-15	-17	120
Nano-Goethite	230	270	-41	-44	-
Trap-Ox Fe-zeolites	780*	780*	-65	-60	250

:- No data

*: sedimentation after suspension in deionized water occurred

Figure 1 Growth rate of *E. coli* after 6h exposure to FerMEG12, Carbo-Iron[®], magnetite, Nano-Goethite and Trap-Ox Fe-zeolites at 0, 50, 500 and 1000 mg/L. Asterisks indicate treatments that differ significantly from controls (ANOVA and Dunnett's test, n=3). Significance levels were set at $P < 0.05$ (*), $P < 0.001$ (**) and $P < 0.0001$ (***).

Figure 2 Root length of radish (*Raphanus sativus*) and ryegrass (*Lolium multiflorum*) exposed to various nominal Fe concentrations from FerMEG12 particles for 6 d. Asterisks indicate treatments that differ significantly from controls (Holm-Sidak, n=3, P<0.05 (*), P<0.001 (**)).

The root elongation of *R. sativus* was reduced by 33 % by FerMEG12 particles at a nominal Fe concentration as low as 10 mg/L. Root elongation was increasingly reduced in a concentration-dependent manner and completely inhibited at 10 g/L (Figure 2, left). The root elongation of *L. multiflorum* was significantly reduced at nominal Fe concentrations >1 g/L, and completely inhibited at 10 g/L (Figure 2, right). The pH of the exposure suspensions at the beginning of the experiment was 6.0 ± 0.5 over the whole concentration range. In contrast, the oxidation reduction potential, measured in exposure suspensions at the beginning of the experiment, was dramatically different among concentrations and ranged from +250 mV (control), +50 mV (0.01-0.1 g/L), to -590 mV (5 and 10 g/L).

Figure 3 Mortality of *L. variegatus* after 24-96 h exposure to FerMEG12 (left axis) and dissolved oxygen (DO) concentration in test beakers after 0-96 h (right axis).

For the *L. variegatus* test, no toxicity was observed at 1 mg/L, however more than 50 % mortality was observed at 5 mg/L and 100 % at 10 mg/L. Mortality was observed already after 24 h, together with a rapid decrease in the dissolved oxygen concentration in test beakers containing FerMEG12 (see Figure 3).

3.3 Technical challenges of ecotoxicity testing of Fe nanomaterials

The higher concentrations of Fe nanomaterials did in several cases influence the measurement principles or assumptions behind the tests. Especially the turbidity of the suspensions caused issues with limited light transmission

through the suspensions. In the *V. fischeri* test, the quantification of the bioluminescence could be influenced by quenching of the emitted light before it reaches the detector. This can easily be measured in a double vial setup that ensures no actual bacterial exposure to the suspension, with the inner vial containing the bacteria. A way to account for this is by spiking the suspension with *V. fischeri* emitting a known amount of bioluminescence. In this way, the added bacteria can act as an internal standard and the effect of quenching can be estimated and corrected for. Using this method, it was clear that the tested materials did quench light emission, giving rise to potential erroneous conclusions if unaccounted for.

For the algal growth inhibition, issues with the quantification of algal biomass also started to appear at higher concentrations (>100 mg/L). At high Fe concentrations the fluorescence spectrum was altered significantly and obfuscated the presence and the size of the chlorophyll peak. During the testing, the turbidity can also prevent the algae to obtain sufficient light for exponential growth, a shading effect that can be difficult to account for (Hjorth et al., 2015).

In the tests with *D. magna* and *L. variegatus*, oxidation, aggregation, precipitation and ultimately sedimentation of Fe resulted in a change of exposure route (which was intended to be through the water phase alone) directly affecting the mobility of *D. magna*. Due to the described stratification of Fe particles, *L. variegatus* was exposed to an increased

concentration, as both the particles and the oligochaeta stay at the bottom of the beaker.

4 Discussion

4.1 Ecotoxicity of Fe nanomaterials

Based on the performed ecotoxicity tests in the present study, only the FerMEG12 particles would be classified as toxic to aquatic organisms in accordance to the CLP regulation. As none of the other tested materials showed toxicity below 100 mg/L, none of them would receive any environmental hazard classification. The highest toxicity of the FerMEG12 particles was observed towards the oligochaeta *L. variegatus*. However particle sedimentation during the test consequently exposed *L. variegatus* to higher Fe concentrations than what was initially dispersed, which could explain why toxicity was observed for *L. variegatus* and not e.g. for *D. magna* which spends more time in the water column. Ageing the particles for 1 h in media alleviated the toxicity, which is in agreement with an earlier study on milled particles reporting low toxicity after oxidation (Köber et al., 2014). Similarly, a recent study on zebrafish found no adverse effects of aged Carbo-Iron[®] (Weil et al., 2015).

The FerMEG12 particles were also tested in the standard algal test with *P. subcapitata* with acute effects evidenced by a decrease in fluorescence right after the onset of the test (data not shown). However, during the incubation period the algal population recovered and exhibited growth rates similar (or higher) than the non-exposed controls. As the only material out of the four tested, FerMEG12 was dispersed in a solvent (ethylene glycol) and although ethylene glycol in itself showed no toxicity when tested, it seems to have preserved the reactivity of FerMEG12's elemental iron. Other studies confirm the toxicity of freshly prepared, non-oxidized nZVI. For instance, Keller et al. (2012) studied the response of microalgae and *D. magna* exposed to NANOFER STAR and 25S. *D. magna* proved the most sensitive with LOEC values of 0.5 mg/L for the NANOFER STAR and NANOFER 25s, compared to a LOEC of 1 mg/L for Fe^{2+} . Whereas the growth of the marine microalga *I. galbana* was inhibited after exposure to NANOFER 25s starting at 3 mg/L (Keller et al., 2012), no effect was observed for NANOFER STAR at concentrations up to 100 mg/L and effects from dissolved Fe did not occur at concentrations lower than 50 mg/L. For the freshwater microalgae, *P. subcapitata*, Fe^{2+} exposure proved the most toxic with a LOEC value of 5 mg/L, which was lower than for any of the particles or Fe^{3+} (Keller et al., 2012).

NANOFER 25s has also been found to affect the growth of the nematode *C. elegans* at 0.5 mg/L, whereas at 5 mg/L a decrease in survival and

reproduction occurred. However, when tested in soil, no toxicity to *C. elegans* was observed for NANOFER 25s at concentrations up to 17 mg/g. On the contrary, their growth and reproduction increased (Saccà et al., 2014). Similarly, the survival of the earthworm, *E. fetida*, was not affected by nZVI even at 3 g/kg, although DNA damage and lipid oxidation was observed (Yirsaw et al., 2016).

Chen et al. (2011) investigated continuous exposure of carboxymethyl cellulose stabilized nZVI (CMC-nZVI) towards medaka fish larvae and concluded that the toxicity was caused by hypoxia, Fe^{2+} toxicity, and ROS-mediated oxidative damage. In their experiment Fe^{2+} proved the most acutely toxic with 100 % mortality at 75 mg/L. In a similar study, Chen et al. (2012) reported Fe^{2+} being the most toxic form of Fe tested, followed by CMC-nZVI, nZVI and lastly the aged nZVI. Yet in Chen et al. (2013) CMC-nZVI had a higher acute toxicity than both Fe^{2+} and aged nZVI.

Marsalek et al. (2012) have reported low aquatic toxicity for NANOFER 25 with $\text{EC}_{50} > 1$ g/L for *D. magna* and >2.5 g/L for fish (*P. reticulate*) and similar values for willows (*S. alba*), duckweed (*L. minor*), and microalgae (*D. subspicatus*). Effects on the cyanobacteria *M. aeruginosa* were observed at the lowest concentrations in the test battery and with an EC_{50} -value of 50 mg/L.

Although there are signs of increased ecotoxicity of nZVI compared to Fe^{2+} , which seems rational due to the additional oxidative capacity of Fe^0 , the

opposite is also sometimes the case. However none of the studies in the scientific literature on Fe nanomaterials have reported a higher toxicity than what has been shown for dissolved Fe (Johnson et al., 2007). It seems likely that varying, and generally lower, bioavailability of nZVI in media has the potential to offset any increase in toxicity compared to dissolved Fe.

4.2 Environmental risk assessment of Fe in surface water

Whereas hazard and risk assessment of Fe nanomaterials is a novel task, the toxic effects of Fe and Fe salts on aquatic life is well described. Fe^{2+} is generally considered bioavailable and can induce toxicity in aquatic organisms, whereas when oxidized to Fe^{3+} it hydrolyses and precipitates out of solution as hydroxides at normal pH, which then can give rise to indirect, physical effects (Vuori, 1995). Ecotoxicity testing of Fe has traditionally made a distinction between total Fe and dissolved Fe, ideally signifying Fe^{2+} , however in practice meaning anything that passes through a 0.45 μm filter, which includes colloidal Fe(III) stabilized mostly by organic material (Vuori, 1995) and Fe(III) complexed by organic ligands. In this way, many tests have already, indirectly, assessed the toxicity of suspended Fe in the nano range. Even for Fe, hazard and risk assessment as well as setting appropriate

environmental quality standards (EQS) has been difficult for decades due to this redox cycling, inorganic speciation, complexation and precipitation.

As an example, the company American Electric Power (AEP) argued in 1983 that the USEPA water quality standard of 1 mg/L for Fe was too low, stating that field data showed that Fe concentrations “need to be far over 1 mg/L to adversely affect” aquatic life, and that toxicity testing is not fit to assess Fe toxicity (Loeffelman et al., 1985). AEP also called for a use of Fe^{2+} , and not total Fe, as the foundation for setting the water quality standard, based on the fact that Fe^{2+} is the bioavailable fraction of Fe.

More recently, Linton et al. (2007) acknowledged that the USEPA “metal policy” generally is to derive “aquatic life criteria” based on the dissolved metal and therefore Fe should be regulated based on Fe^{2+} toxicity. Yet, it makes sense to use field data on total Fe, as lab-based toxicity testing of Fe is poor at assessing colloidal and indirect effects such as the impact on respiration and food consumption. With reference to a review by Vouri (1995) who states “the effect of Fe on aquatic animals and their habitats are mainly indirect”, Linton et al. (2007) argue that field studies therefore would do a better job of assessing the overall environmental impact of Fe. Accordingly, the assessment still has a foundation in field observations partly due to this issue of indirect effects (Linton et al., 2007). Based on additional field data, Linton et al. (2007) however proposed a new and differentiated

EQS of 0.21 mg/L for sensitive groups and 1.74 mg/L for “slight to moderate changes” in the aquatic community.

In Europe, the Environment Agency in England proposed a new EQS for Fe in 2007 under the Water Framework Directive (Johnson et al., 2007). A long term PNEC of 16 µg/L was derived, based on a NOEC value of 0.16 mg/L obtained in a 21-d study on *D. magna*. Similarly, a 96 h study on brook trout (*S. fontinalis*) with a LC₅₀ value of 0.41 mg/L gave rise to a short term PNEC of 41 µg/L. They rationalized the use of the considerably lower PNEC values with the emergence of new data as well as the fact that the old EQS was based on field data and not on standardized toxicity tests.

In response to this, Crane et al. (2007) stated that 16 µg/L was “substantially below concentrations associated with impaired invertebrate assemblages in the field”. Based on their analysis of data from 253 sites in England and Wales, Crane et al. (2007) proposed an EQS between 43-250 µg/L based on dissolved Fe. In 2012, the UK Technical Advisory Group proposed an EQS of 0.73 mg/L total Fe, based on field data, in a very thorough review (Peters et al., 2012). The suggested EQS from the Environment Agency of 16 µg/L was described as well below background levels and therefore “not adopted for regulatory use”.

In accordance with Linton et al. (2007), Peters et al. (2012) state that the effects of Fe are difficult to isolate and, in contrast to most metals, we cannot just focus on the dissolved fraction as there is also a physical effect from the

total Fe, which perhaps could even be dominating. This line of reasoning also advocates for the use of total Fe as the dose metric for ecotoxicity studies on Fe nanomaterials.

4.3 Applicability of standardized ecotoxicity tests for hazard identification of Fe nanomaterials

As shown above, the current consensus seems to move away from using standardized ecotoxicity tests on Fe for risk assessment purposes; rather the use of field data and mesocosm studies are encouraged. Peters et al. (2012) point towards the issue of Fe solubility as well as the issue with assessing physical effects as the key points against using standardized ecotoxicity testing of Fe. Wess (2015) also questions the adequacy of ecotoxicity tests on Fe to inform risk assessment as they fail to uphold various criteria for assessing causation, e.g. issues with establishing dose response relationships and incoherence with field data. If these ecotoxicity tests are not suitable for Fe salts due to precipitation and exposure control issues, then clearly they are not suitable to assess Fe nanomaterials, which by definition are not dissolved entities.

However, having the test limitations and challenges in mind, such as the ones mentioned in section 3.3, as well as the general testing considerations in nanoecotoxicology (Petersen et al., 2014; Skjolding et al., 2016) and indirect

physical effects (Sørensen et al., 2015), standardized ecotoxicity tests can still provide valuable information, as a screening and ranking tool for hazard identification. Trying to overcome these limitations will sometimes result in deviations from the standard tests, which at times are necessary to obtain meaningful data. Additionally, the information generated from standardized ecotoxicity tests is still required by regulatory agencies to achieve market access.

On the other hand, as shown for Fe salts, standardized ecotoxicity tests are challenged in terms of their usefulness and accuracy for site-specific and case oriented risk assessments, and it is recommended to rely more on field data when assessing the environmental impact of Fe (EC, 2011). The relevance of standard organisms for site-specific risk assessment is questionable for remediation cases, as the extrapolation value from these organisms to ecosystems may be low. In addition, the transformation of the pristine material to the oxidized form expected in the environment and the corresponding change in toxicity is also better assessed in the field. As such, it makes sense to rely less on laboratory ecotoxicity testing and instead incorporate mesocosm and field data into site-specific risk assessment of Fe nanomaterials.

5 Conclusion and recommendations

Ecotoxicity testing of four nanomaterials engineered for sub-surface remediation revealed low toxicity for all Fe oxides materials as well as Carbo-Iron[®], and the results do not lead to any hazard classification according to current EU regulation. FerMEG12 was the only material exhibiting toxicity towards bacteria and plants at 50 mg/L and oligochaetes at 5 mg/L. Standard ecotoxicity testing of nanoparticles has in general proven technically difficult and it may be questioned whether proper hazard identification of engineered nanoparticles needed for environmental risk assessment is currently feasible. Aggregation, agglomeration, sedimentation, shading, and other physical effects are known to confound the measuring principles behind the tests and these interferences were also observed for the tested particles. This was pronounced for tests on algae, bacteria, and crustaceans and requires inclusion of additional controls to ensure a correct data interpretation. While Fe⁰ nanomaterials have the potential to be toxic at low concentrations, a potential environmental impact downstream of the injection of Fe nanomaterials seems more likely to originate from the large amount of Fe injected in *in-situ* remediation, than from novel particle related effects, especially since Fe is abundant in nature as particulate matter. In conclusion we recommend:

- Testing concentrations up to 100 mg/L is relevant for hazard identification and classification purposes. Testing Fe nanoparticles at higher concentrations not only decreases the environmental relevance, but also increases the influence of physical effects such as turbidity and concentration-dependent agglomeration. Therefore, the main focus in testing should be on concentrations <100 mg/L, and care should be taken when conducting ecotoxicological testing of Fe nanomaterials at higher concentrations.
- For Fe nanomaterials, agglomeration and sedimentation challenge the validity of the standard test setups for which a constant exposure during incubation is required. These issues are currently under scrutiny in the OECD WPMN and in several EU projects (Lynch, 2016). We recommend the use of these tests for hazard identification and ranking, where these tests still provide valuable information.
- We recommend studying the effects of Fe nanomaterials in more environmentally realistic conditions to support site-specific hazard assessment. E.g. through testing of relevant organisms, media and more complex testing systems closer related to the field, as the scope of standardized ecotoxicity testing is limited and not designed to assess the indirect effects of Fe exposure.

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Highlights

- This study summarizes the outcome of nanoecotoxicity testing in NanoRem.
- We assessed four novel engineered nanomaterials in an ecotoxicological test battery.
- Only one of the tested materials gave rise to toxicity below 100 mg/L.
- Standardized testing is inadequate to inform site-specific risk assessment.