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

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15

## 16 Abstract

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

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

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

39

## 40 1 Introduction

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

44 Mueller et al., 2012). Especially iron (Fe) based nanomaterials have shown a  
45 potential for remediation due to a larger specific surface area and  
46 corresponding increased reactivity compared to micro-scale and larger Fe  
47 materials traditionally used for remediation of contaminated sites (Wang and  
48 Zhang, 1997). Nanoscale zerovalent iron (nZVI) has received most of the  
49 attention as it is highly reactive compared to the bulk ZVI used in permeable  
50 reactive barriers (Henderson and Demond, 2007). Remediation with nZVI has  
51 been claimed to represent a faster, cheaper and a potentially more effective  
52 treatment option than current *ex situ* and *in situ* methods (Yan et al., 2013).  
53 However, nanomaterials engineered to remediate polluted soil and  
54 groundwater may constitute a risk to the environment as they are injected into  
55 the subsurface in large quantities (Grieger et al., 2010). As such this could  
56 represent a worst case scenario when considering possible negative  
57 environmental effects of manufactured nanomaterials. Nanoremediation in  
58 general seems associated with high uncertainty both in relation to its potential  
59 environmental risks, but also towards its field scale efficacy (Grieger et al.,  
60 2015). Uncertainty with regards to the potential environmental impacts of Fe  
61 nanomaterials hampers their use and has partly been the reason for the  
62 limited implementation of Fe nanomaterials in remediation (Bardos et al.,  
63 2014), although no major environmental impacts have been reported in the  
64 first decade of field deployments with Fe nanomaterials (Mueller et al.,  
65 2012).

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

74 A range of Fe nanomaterials has been developed in the European FP7 project  
75 NanoRem (Taking Nanotechnological Remediation Processes from Lab Scale  
76 to End User Applications for the Restoration of a Clean Environment, for  
77 more information see nanorem.eu) in order to extend the spectrum of  
78 treatable soil and groundwater contaminants from halogenated organics to  
79 non-halogenated substances and non-reducible metals. Contrary to the three  
80 materials mentioned above, the potential ecotoxicity of the materials  
81 developed in NanoRem have not previously been tested. These materials are  
82 currently available on the international market (see Table 1). Common for all  
83 materials is that if they are to be used in field-scale remediation, their  
84 production volume will easily reach 1 metric ton per year (Mueller et al.,  
85 2012). In this case, they will have to be registered under the European  
86 chemical legislation REACH, which will be accompanied with data  
87 requirements on ecotoxicity. The data generated will feed into the general

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

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

107

## 108 2 Materials and Methods

### 109 2.1 Nanomaterials

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

#### 113 2.1.1 Dispersion of nanomaterials for toxicity testing

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

119 *Carbo-Iron*<sup>®</sup> For 100 mL of a stock suspension at 10 g/L, 20 mL of a 10 g/L  
120 carboxymethyl cellulose (CMC) solution was added to 80 mL of test medium  
121 and degassed with N<sub>2</sub> for an hour. Then, 1 g of test material was added to the  
122 solution under N<sub>2</sub> flow, and mixed for 10 min with a high-shear mixer.  
123 Dilution series were prepared under regular aerobic conditions and used right  
124 away.

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



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

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

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

136

## 137 2.2 Characterization of stock suspensions

138 Dynamic Light Scattering (DLS) measurements of aqueous suspensions  
139 (deionized water) from 10 mg/L to 10 g/L test material were performed on a  
140 Malvern Zetasizer ZS (Malvern instruments Ltd, Worcestershire, UK)  
141 equipped with a laser source at a wavelength of 633 nm. Zeta-averaged  
142 hydrodynamic diameters and size distributions were determined using the  
143 “multiple narrow modes (high resolution)” algorithm supplied by Malvern.  
144 Measurements were done in triplicates of 5 runs with autocorrelation  
145 functions of 10 seconds. The same instrument was used for the measurements  
146 of electrophoretic mobility and the Smoluchowski approximation was used  
147 for determining zeta-potentials. Three measurements with 5 runs per  
148 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).

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153 **Table 1** Characterization of the pristine Fe nanomaterials. Magnetite is not used for remediation in NanoRem but was chosen as a  
 154 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 <sup>2</sup> /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 <sup>®</sup>	Composite of activated carbon and zero-valent Fe	Field tested and commercially available	Adsorption + Reduction	Powder	30.3 % Fe <sub>tot</sub> 20.5 % Fe <sup>0</sup> 13.1 % Fe <sub>3</sub> O <sub>4</sub> 55±1% C <sub>tot</sub>	13440 ±20	594
Magnetite	Fe oxides (Fe <sub>3</sub> O <sub>4</sub> )	Precursor for NANO FER STAR	-	Powder	Fe <sub>3</sub> O <sub>4</sub>	-	-
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

155

156 The light source was a solid-state, single-mode laser diode (radiation output  
157 max power  $<50\mu\text{W}$ , 635nm continuous wave, max power  $< 35\text{mW}$ ). The  
158 standard camera Marlin F-033B (Allied Vision Technologies GmbH,  
159 Stadtroda, Germany) was used. All data were analyzed using the instrument  
160 software (NanoSight™ version 2.2). The analysis with NTA was done on 7  
161 videos with 1 min length each. The solution oxidation-reduction potential and  
162 pH were measured in all exposure suspensions at the beginning and the end  
163 of the tests. Total Fe concentration in stock suspensions was measured by  
164 ICP-OES (Perkin Elmer, Optima 5300 DV) following microwave assisted  
165 digestion under acidic conditions (3.7 % HCl).

166

## 167 2.3 Ecotoxicological test battery

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

175

176 **Table 2** Organisms and testing endpoints of the eight ecotoxicity tests in the test  
177 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 <sup>a</sup>
Plant	<i>Raphanus sativus</i> , <i>Lolium multiflorum</i>	6 d	Root elongation	OECD 208

178 <sup>a</sup> modified to short term water-phase exposure

179

## 180 3 Results

### 181 3.1 Characterization

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

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

196

## 197 3.2 Ecotoxicity

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

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

210

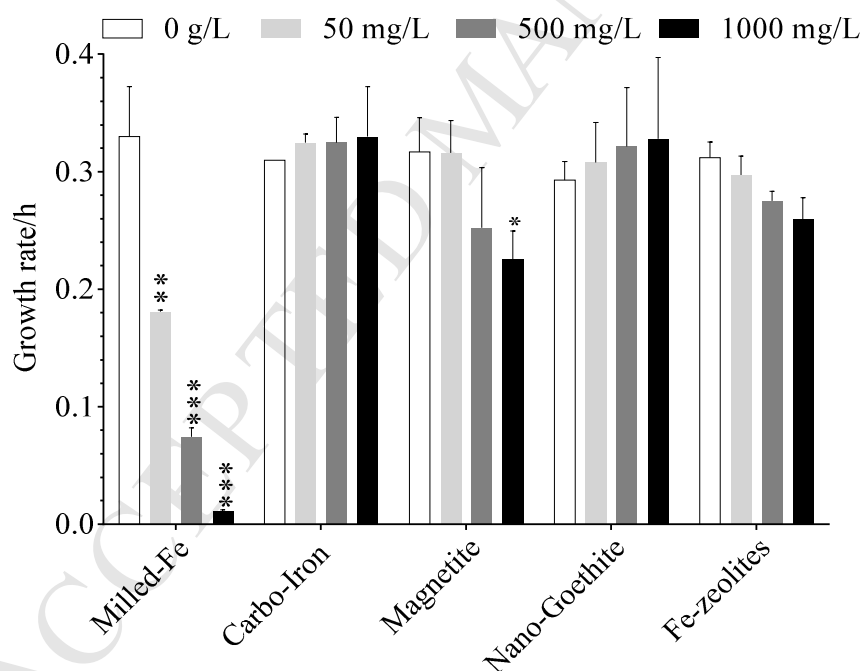
211 **Table 3** Characterization of the four tested nanomaterials dispersed in deionized  
 212 water at 100 mg/L. Samples were characterized 2 and 144 h after dispersion and  
 213 analyzed by DLS for hydrodynamic diameter and zeta-potential. NTA analysis was  
 214 performed in samples 2 h after dispersion.

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 <sup>®</sup>	1300	500	-15	-17	120
Nano-Goethite	230	270	-41	-44	-
Trap-Ox Fe-zeolites	780*	780*	-65	-60	250

215 -: No data

216 \*: sedimentation after suspension in deionized water occurred

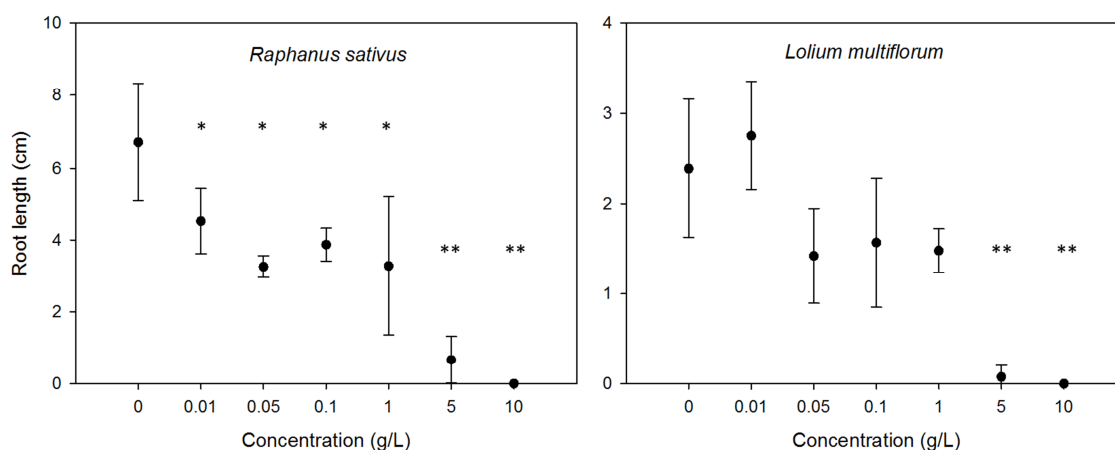
217



218

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

224



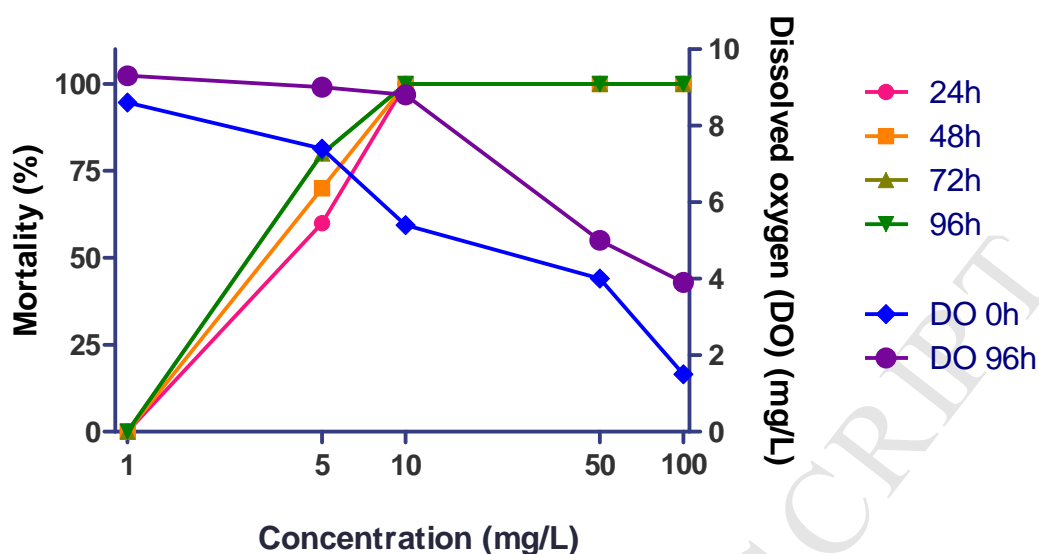
225

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

230

231 The root elongation of *R. sativus* was reduced by 33 % by FerMEG12  
 232 particles at a nominal Fe concentration as low as 10 mg/L. Root elongation  
 233 was increasingly reduced in a concentration-dependent manner and  
 234 completely inhibited at 10 g/L (Figure 2, left). The root elongation of *L.*  
 235 *multiflorum* was significantly reduced at nominal Fe concentrations >1 g/L,  
 236 and completely inhibited at 10 g/L (Figure 2, right). The pH of the exposure  
 237 suspensions at the beginning of the experiment was  $6.0 \pm 0.5$  over the whole  
 238 concentration range. In contrast, the oxidation reduction potential, measured  
 239 in exposure suspensions at the beginning of the experiment, was dramatically  
 240 different among concentrations and ranged from +250 mV (control), +50 mV  
 241 (0.01-0.1 g/L), to -590 mV (5 and 10 g/L).





242  
 243 **Figure 3** Mortality of *L.variegatus* after 24-96 h exposure to FerMEG12 (left axis)  
 244 and dissolved oxygen (DO) concentration in test beakers after 0-96 h (right axis).

245  
 246 For the *L. variegatus* test, no toxicity was observed at 1 mg/L, however more  
 247 than 50 % mortality was observed at 5 mg/L and 100 % at 10 mg/L. Mortality  
 248 was observed already after 24 h, together with a rapid decrease in the  
 249 dissolved oxygen concentration in test beakers containing FerMEG12 (see  
 250 Figure 3).

251

### 252 3.3 Technical challenges of ecotoxicity testing of 253 Fe nanomaterials

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

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

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

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

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

281

## 282 4 Discussion

### 283 4.1 Ecotoxicity of Fe nanomaterials

284 Based on the performed ecotoxicity tests in the present study, only the  
285 FerMEG12 particles would be classified as toxic to aquatic organisms in  
286 accordance to the CLP regulation. As none of the other tested materials  
287 showed toxicity below 100 mg/L, none of them would receive any  
288 environmental hazard classification. The highest toxicity of the FerMEG12  
289 particles was observed towards the oligochaeta *L. variegatus*. However  
290 particle sedimentation during the test consequently exposed *L. variegatus* to  
291 higher Fe concentrations than what was initially dispersed, which could  
292 explain why toxicity was observed for *L. variegatus* and not e.g. for *D.*  
293 *magna* which spends more time in the water column. Ageing the particles for  
294 1 h in media alleviated the toxicity, which is in agreement with an earlier  
295 study on milled particles reporting low toxicity after oxidation (Köber et al.,  
296 2014). Similarly, a recent study on zebrafish found no adverse effects of aged  
297 Carbo-Iron<sup>®</sup> (Weil et al., 2015).

298 The FerMEG12 particles were also tested in the standard algal test with *P.*  
299 *subcapitata* with acute effects evidenced by a decrease in fluorescence right  
300 after the onset of the test (data not shown). However, during the incubation  
301 period the algal population recovered and exhibited growth rates similar (or  
302 higher) than the non-exposed controls. As the only material out of the four  
303 tested, FerMEG12 was dispersed in a solvent (ethylene glycol) and although  
304 ethylene glycol in itself showed no toxicity when tested, it seems to have  
305 preserved the reactivity of FerMEG12's elemental iron. Other studies confirm  
306 the toxicity of freshly prepared, non-oxidized nZVI. For instance, Keller et  
307 al. (2012) studied the response of microalgae and *D. magna* exposed to  
308 NANOFER STAR and 25S. *D. magna* proved the most sensitive with LOEC  
309 values of 0.5 mg/L for the NANOFER STAR and NANOFER 25s, compared  
310 to a LOEC of 1 mg/L for Fe<sup>2+</sup>. Whereas the growth of the marine microalga *I.*  
311 *galbana* was inhibited after exposure to NANOFER 25s starting at 3 mg/L  
312 (Keller et al., 2012), no effect was observed for NANOFER STAR at  
313 concentrations up to 100 mg/L and effects from dissolved Fe did not occur at  
314 concentrations lower than 50 mg/L. For the freshwater microalgae, *P.*  
315 *subcapitata*, Fe<sup>2+</sup> exposure proved the most toxic with a LOEC value of 5  
316 mg/L, which was lower than for any of the particles or Fe<sup>3+</sup> (Keller et al.,  
317 2012).

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

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

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

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

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

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

347

## 348 4.2 Environmental risk assessment of Fe in surface 349 water

350 Whereas hazard and risk assessment of Fe nanomaterials is a novel task, the  
351 toxic effects of Fe and Fe salts on aquatic life is well described.  $\text{Fe}^{2+}$  is  
352 generally considered bioavailable and can induce toxicity in aquatic  
353 organisms, whereas when oxidized to  $\text{Fe}^{3+}$  it hydrolyses and precipitates out  
354 of solution as hydroxides at normal pH, which then can give rise to indirect,  
355 physical effects (Vuori, 1995). Ecotoxicity testing of Fe has traditionally  
356 made a distinction between total Fe and dissolved Fe, ideally signifying  $\text{Fe}^{2+}$ ,  
357 however in practice meaning anything that passes through a 0.45  $\mu\text{m}$  filter,  
358 which includes colloidal Fe(III) stabilized mostly by organic material (Vuori,  
359 1995) and Fe(III) complexed by organic ligands. In this way, many tests have  
360 already, indirectly, assessed the toxicity of suspended Fe in the nano range.  
361 Even for Fe, hazard and risk assessment as well as setting appropriate

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

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

371 More recently, Linton et al. (2007) acknowledged that the USEPA “metal  
372 policy” generally is to derive “aquatic life criteria” based on the dissolved  
373 metal and therefore Fe should be regulated based on  $\text{Fe}^{2+}$  toxicity. Yet, it  
374 makes sense to use field data on total Fe, as lab-based toxicity testing of Fe is  
375 poor at assessing colloidal and indirect effects such as the impact on  
376 respiration and food consumption. With reference to a review by Vouri  
377 (1995) who states “the effect of Fe on aquatic animals and their habitats are  
378 mainly indirect”, Linton et al. (2007) argue that field studies therefore would  
379 do a better job of assessing the overall environmental impact of Fe.  
380 Accordingly, the assessment still has a foundation in field observations partly  
381 due to this issue of indirect effects (Linton et al., 2007). Based on additional  
382 field data, Linton et al. (2007) however proposed a new and differentiated

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

385 In Europe, the Environment Agency in England proposed a new EQS for Fe  
386 in 2007 under the Water Framework Directive (Johnson et al., 2007). A long  
387 term PNEC of 16 µg/L was derived, based on a NOEC value of 0.16 mg/L  
388 obtained in a 21-d study on *D. magna*. Similarly, a 96 h study on brook trout  
389 (*S. fontinalis*) with a LC<sub>50</sub> value of 0.41 mg/L gave rise to a short term PNEC  
390 of 41 µg/L. They rationalized the use of the considerably lower PNEC values  
391 with the emergence of new data as well as the fact that the old EQS was  
392 based on field data and not on standardized toxicity tests.

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

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



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

408

### 409 4.3 Applicability of standardized ecotoxicity tests 410 for hazard identification of Fe nanomaterials

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

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

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

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

## 445 5 Conclusion and recommendations

446 Ecotoxicity testing of four nanomaterials engineered for sub-surface  
447 remediation revealed low toxicity for all Fe oxides materials as well as  
448 Carbo-Iron<sup>®</sup>, and the results do not lead to any hazard classification  
449 according to current EU regulation. FerMEG12 was the only material  
450 exhibiting toxicity towards bacteria and plants at 50 mg/L and oligochaetes at  
451 5 mg/L. Standard ecotoxicity testing of nanoparticles has in general proven  
452 technically difficult and it may be questioned whether proper hazard  
453 identification of engineered nanoparticles needed for environmental risk  
454 assessment is currently feasible. Aggregation, agglomeration, sedimentation,  
455 shading, and other physical effects are known to confound the measuring  
456 principles behind the tests and these interferences were also observed for the  
457 tested particles. This was pronounced for tests on algae, bacteria, and  
458 crustaceans and requires inclusion of additional controls to ensure a correct  
459 data interpretation. While Fe<sup>0</sup> nanomaterials have the potential to be toxic at  
460 low concentrations, a potential environmental impact downstream of the  
461 injection of Fe nanomaterials seems more likely to originate from the large  
462 amount of Fe injected in *in-situ* remediation, than from novel particle related  
463 effects, especially since Fe is abundant in nature as particulate matter. In  
464 conclusion we recommend:

- 465 • Testing concentrations up to 100 mg/L is relevant for hazard identification  
466 and classification purposes. Testing Fe nanoparticles at higher concentrations  
467 not only decreases the environmental relevance, but also increases the  
468 influence of physical effects such as turbidity and concentration-dependent  
469 agglomeration. Therefore, the main focus in testing should be on  
470 concentrations <100 mg/L, and care should be taken when conducting  
471 ecotoxicological testing of Fe nanomaterials at higher concentrations.
- 472
- 473 • For Fe nanomaterials, agglomeration and sedimentation challenge the validity  
474 of the standard test setups for which a constant exposure during incubation is  
475 required. These issues are currently under scrutiny in the OECD WPMN and  
476 in several EU projects (Lynch, 2016). We recommend the use of these tests  
477 for hazard identification and ranking, where these tests still provide valuable  
478 information.
- 479
- 480 • We recommend studying the effects of Fe nanomaterials in more  
481 environmentally realistic conditions to support site-specific hazard  
482 assessment. E.g. through testing of relevant organisms, media and more  
483 complex testing systems closer related to the field , as the scope of  
484 standardized ecotoxicity testing is limited and not designed to assess the  
485 indirect effects of Fe exposure.

486

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497

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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.