Ecotoxicological effects on earthworms of fresh and aged nano-sized zero-valent iron (nZVI) in soil

by Yehia S. El-Temsah and Erik J. Joner*

Yehia.sayed.el-temsah@bioforsk.no, Erik.Joner@bioforsk.no

Norwegian Institute for Agricultural and Environmental Research (Bioforsk),
Soil and Environment Division,
Frederik A. Dahls vei 20,
NO-1432 Ås,
Norway.

*Corresponding author
Phone. +47 928 33 168
Fax: +47 63 00 94 10

Ecotoxicological effects on earthworms of fresh and aged nano-sized zero-valent iron (nZVI) in soil

Yehia S. El-Temsah and Erik J. Joner

Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Soil and Environment Division, Fredrik A. Dahls vei 20, NO-1432 Ås, Norway.

Abstract.

Although nano-sized zero-valent iron (nZVI) has been used for several years for remediation of contaminated soils and aquifers, only a limited number of studies have investigated secondary environmental effects and ecotoxicity of nZVI to soil organisms. In this study we therefore measured the ecotoxicological effects of nZVI coated with carboxymethyl cellulose on two species of earthworms, *Eisenia fetida* and *Lumbricus rubellus,* using standard OECD methods with sandy loam and artificial OECD soil. Earthworms were exposed to nZVI concentrations ranging from 0 to 2000 mg nZVI kg soil^{-1} added freshly to soil or aged in non-saturated soil for 30 days prior to exposure. Regarding avoidance, weight changes and mortality, both earthworm species were significantly affected by nZVI concentrations ≥500 mg kg^{-1} soil. Reproduction was affected also at 100 mg nZVI kg^{-1}. Toxicity effects of nZVI were reduced after aging with larger differences between soils compared to non-aged soils. We conclude that doses ≥500 mg nZVI kg^{-1} are likely to give acute adverse effects on soil organisms, and that effects on reproduction may occur at significantly lower concentrations.

Keywords: Earthworms, ecotoxicological effects, iron, nanoparticles, nZVI, remediation.

Abbreviations: nZVI - nano-sized zero-valent iron.

1. Introduction

During the last decade, nano-sized zero-valent iron (nZVI) has been tested and used to remediate contaminated soil and groundwater, and has received attention due to its cost efficient degradation or sequestration of environmental pollutants (Chang and Kang, 2009). Remediation of both organic and inorganic contaminants in soil and water has been attempted, including polycyclic aromatic hydrocarbons (PAHs), halogenated organic compounds, pesticides, metalloids and heavy metals (Zhang, 2003; Chang and Kang, 2009; Elliott et al., 2009; Park et al., 2009). The success of remediation and substantial cost reduction compared to *ex-situ* treatments involving pump and treat or excavation has led to prospects for widespread use in the years to come (Cook, 2009; Karn et al., 2009). Yet, ethical and environmental concern for organisms living in soil and surface water has led to questions of possible negative secondary effects which have scarcely been addressed so far (Keane, 2009). Depending on treatment schemes and site-specific characteristics like soil depth and texture, movement of water and pollutants, different organisms are more or less likely to be exposed to nZVI or its transformation products.

So far, only a limited number of studies have investigated the toxicity or ecotoxicity of nZVI. In an aquatic system, Chen et al. (2011) studied the effects of nZVI on antioxidant enzyme activities and lipid peroxidation in Medaka (*Oryzias latipes*) and found enhanced reactive oxygen species (ROS) formation during oxidation of reduced iron. Cullen et al. (2011) studied the impact of polyacrylic acid-coated nano- and micron-sized zero-valent iron particles on microbially derived soil enzyme activities in uncontaminated soil, and found no negative effects of 10 g kg^{-1} of nZVI (or micron-sized Fe^0) on the soil dehydrogenase and hydrolase activity, but a partial inhibitory effect on bacterial ammonium oxidation. Two other

studies on toxic effects to the gut inhabiting bacterium *Escherichia coli* showed that nZVI particles had a bactericidal effect at concentrations above 9 mg L^{-1}, under anaerobic conditions (Lee et al., 2008) and at ≤70 mg L^{-1} under aerobic conditions (Auffan et al., 2008).

Aging and oxidation may affect toxicity of nZVI, as shown by Phenrat et al. (2009) who assessed oxidative stress in rodent brain cells. When comparing freshly prepared nZVI particles, partially oxidized nZVI (aged >11 months), and pure iron oxide (magnetite), they demonstrated decreasing toxicity with increasing degree of Fe oxidation.

All the studies cited above have been conducted in simplified systems without taking into account the effects of contact with soil. Since nZVI is intended for use in soil, there is an evident need to evaluate the potential toxicity of nZVI both on soil organisms and in the presence of soil. Among soil organisms, earthworms play a key role in terrestrial ecosystems by recycling organic matter and mineral nutrients and maintaining soil structure (Edwards and Bohlen, 1996). They may also represent up to 80% of the total soil biomass (Rombke et al., 2005). For these reasons, earthworms are common test organisms in soil ecotoxicity studies (Spurgeon et al., 2003b) and may be used to assess bioavailability of potentially hazardous materials in soil (Conder et al., 2001; Lanno et al., 2004; Ma, 2005). While these and numerous other studies have focused on traditional metal pollution, recent studies have used earthworms to assess the specific properties of engineered nanoparticles (ENPs) with respect to both toxicity and bioavailability (Unrine et al., 2010a; Unrine et al., 2010b; Coutris et al., 2011). So far, testing of ENPs for negative effects on soil invertebrates have only considered metal nanoparticles with stable oxidation status, whereas redox active ENP like Fe^0 have not been assessed.

Ecotoxicological effects on earthworms can range from mild stress reactions, via non lethal effects seen on genetic, physiological or reproductive endpoints, to acute toxicity with high lethality. Avoidance is a behavioral response that may indicate anything from mildly

adverse conditions to weakly or moderately toxic properties of poorly mobile compounds (Capowiez et al., 2005; Zhou et al., 2008). The most common approach to sub-lethal earthworm toxicity is however the assessment of reproduction and growth, which can provide responses ranging from mildly negative effects on reproduction to partial lethality in adults. Such tests have been standardized (e.g. by the OECD) using artificial growth media, but similar tests may also be carried out with natural soils, rendering them more useful for in the context of exposure in specific soil types.

The objectives of the present study were to determine the potential toxic effects of nZVI to earthworms using both standard OECD methods and similar tests in real soil. We also wanted to compare the effects of nZVI on two different earthworm species; *Eisenia fetida* commonly used in standard tests, and *Lumbricus rubellus*. Thus, we compared avoidance behavior in soils containing different concentrations of nZVI with non-amended soil. Further we assessed mortality after exposure to nZVI over periods from 14 to 28 d, and effects on reproduction and growth of earthworms. Additionally, we wanted to compare the effects of freshly added and aged nZVI in soil to determine whether or not the observed negative effects of nZVI were ephemeral or persistent in soil.

2. Materials and methods

2.1. Test organisms

The epigeic earthworms *Eisenia fetida* and *Lumbricus rubellus* (*Lumbricidae*) were purchased from BVC Holland (Surhuisterveen, The Netherlands). The earthworms were placed in plastic boxes containing a mixture of sphagnum peat (30% by dry weight), horse manure (1% d wt), and soil (69% d wt, sandy soil) as a substrate, and moistened regularly (70% of water holding capacity). The culture was maintained at temperature 20±2 °C and a photoperiod of 16:8 h (light:dark). Three days before the beginning of the test, adult worms of

E. fetida and *L. rubellus* with an average weight of 0.6–1.4 g, and 0.4-0.8 g, respectively, were selected and placed in the test soil (sandy loam soil or OECD soil) for acclimatization.

2.2. Soils

Two soils were used in this experiment. First, we used a sandy loam from Gardermoen, Akershus county, S.E. Norway, air-dried and sieved (<2 mm) before use. This soil was subjected to standard soil analyses (Schinner et al., 1996) and had 1.1% organic matter, a $pH_{(water)}$ of 5.8 and a water holding capacity (WHC) of 41%. Further, the mineral fraction consisted of 85% sand, 11% silt and 4% clay. The second soil was an artificial standard soil (OECD, 1984) commonly used for earthworm toxicity tests. The OECD soil was prepared from a mixture of 10% finely ground sphagnum peat (pH 5.5 to 6.0, no visible plant remains), 20% kaolinite clay, 69% air-dried quartz sand (dominant fine sand with >50% with a particle size of 0.05 to 0.2 mm) and 1% calcium carbonate (bringing the pH to 6.0 ± 0.5). These components were mixed thoroughly and de-ionized water added to give an overall moisture content of approx. 50% of WHC.

2.3. Synthesis and characterization of nZVI

Nano-sized zero-valent iron stabilized with carboxymethyl cellulose (CMC) was prepared using the borohydride method with ferrous ion, as described by He et al. (2010), but without using Pd. Briefly, a $FeSO_4$ $7H_2O$ stock solution was prepared immediately before use and mixed with the stabilizer solution (CMC) to yield a desired concentration of Fe^{2+} and CMC. The mixture was shaken for 15 min to ensure formation of Fe^{2+}-CMC complexes. ZVI nanoparticles were then formed by reducing Fe^{2+} ions using a borohydride solution (introduced at 5 mL min^{-1}) at a BH_4^-/Fe^{2+} molar ratio of 2.0. To ensure efficient use of BH_4^-, the suspension was shaken until gas (hydrogen) evolution ceased. Immediately after

preparation, size of nZVI particles suspended in pure water was determined using high resolution transmission electron microscopy (HR-TEM; JEM-2011; Jeol, Japan, operating at 200 keV). A drop of the nZVI suspension was loaded on a TEM grid and dried in laminar flow fume hood. Particle size distribution, hydrodynamic diameter and zeta potential of the nZVI suspension were also determined by dynamic light scattering (DLS) and phase analysis light scattering (PALS), respectively, using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., England). Transmission electron micrographs showed that nZVI particle size were between 20 and 100 nm in diameter, while DLS analysis show that particle size were between 178 and 424 nm (results not shown). Zeta potential of the aqueous nZVI suspension was -42.8 mV. The differences between TEM and DLS size analysis were likely due to particle aggregation during DLS analysis, which continuously increased with time.

2.4. Avoidance tests

The avoidance test was performed in accordance with a standard ISO guideline protocol (ISO, 2005). Rectangular plastic containers (190-160 mm) were used, and a removable plastic wall was used to separate the soil in two compartments of 200 g soil (d wt) during preparation of the experiment. The two soils described above (sandy loam soil and OECD soil) were used in each of two series of experiments. Unamended soil (control) was placed in one compartment, and the soil recently mixed with either of the different concentrations of nZVI was placed in the opposite compartment. Soil humidity was adjusted to 60% of WHC prior to preparation of the experiment. The concentrations of nZVI in the soils were 100, 250, 500, 750, 1000 and 1500 mg kg^{-1} dry soil. Three replicates of each concentration were prepared.

Ten earthworms were then placed on the soil surface in each container, at the interface dividing the two compartments, and the containers covered with perforated transparent lids.

169 Worms were left to migrate between the two differently treated soil compartments for 48 h.
170 At this time, the differently treated soils were separated by re-inserting the removable wall at
171 the interface between the two compartments, and the number of earthworms in each
172 compartment counted. Worms that were cut by splitting the two compartments were
173 considered as being in the soil in which the worm's head was located. A correctly performed
174 avoidance test (see e.g. Hund-Rinke and Wiechering, 2001) needs to fulfill certain criteria to
175 be considered valid: First, there should be a random distribution of earthworms between the
176 two compartments in the containers with only control soil. Further, mortality in these controls
177 must be <20%. The percentage of avoidance was calculated following the equation: %
178 avoidance = ((E−T)/E)×100, where E is the expected number of worms in the control soil
179 assuming homogeneous distribution of earthworms between the two compartments (if N=10,
180 then E=5), and T is the average number of worms counted in the soil with the test compound
181 (Marques et al., 2009).

182 Avoidance from soil containing the test compound was indicated by a positive value
183 and preference for the treated soil by a negative value. Two types of analysis may be used for
184 indicating a response: A threshold value and statistical analysis. The threshold value considers
185 that the tested soil has an impaired habitat function when ≥ 80% of the worms migrate to the
186 control soil, which corresponds to >60% avoidance, as suggested by ISO (2005).

187 For statistical analysis, a one way analysis of variance (ANOVA) was used to assess
188 the differences in avoidance between nZVI treatments and controls. The latter was considered
189 as having 0% avoidance (Marques et al., 2009). Probit regression analysis (EPA Probit
190 analysis, v. 1.5) was used to determine EC50 values (50% effect concentration) using %
191 avoidance at the different exposure concentrations.

192

193 **2.5. Mortality and reproduction tests**

194 Acute and chronic toxicity tests were performed according to OECD guidelines using
195 the two soils described above. Two hundred grams of soil were filled into 400 mL plastic
196 containers amended with nZVI suspensions to bring the moisture content to 50% of WHC and
197 obtain nZVI concentrations of 0, 100, 250, 500, 750 and 1000 mg kg soil^{-1}. Three replicate
198 containers were prepared for each earthworm species and nZVI concentration. The soil in the
199 containers was mixed well and three earthworms were weighed (average weight 0.8-1.5 g and
200 0.7-1.2 g for *E. fetida* and *L. rubellus*, respectively) and introduced to each container. The
201 containers were covered with perforated transparent lids and 2 g of freshly moistened horse
202 manure (previously dried and frozen, and originating from a non-medicated horse) was added
203 to the surface of the soil in each container at the start of the experiment and then replenished
204 once a week for the duration of the test (4 weeks). Remaining manure from previous feedings
205 was removed from the soil surface. Mortality (failure to react to a gentle mechanical stimulus)
206 was recorded after 14 d (acute OECD test). Surviving earthworms were returned to their
207 respective containers and incubated for 2 more weeks to assess chronic effects on growth and
208 reproduction. After a total exposure time of 4 weeks adult earthworms, cocoons and juveniles
209 were extracted, counted and weighed.
210
211 **2.6. Aging effects on nZVI toxicity**
212 To test the effect of aging on the toxicity of nZVI to earthworms, the soil with the same
213 humidity and concentrations of nZVI (100-2000 mg kg^{-1}) were prepared and left for 30 d to
214 allow atmospheric oxygen and soil particles to react with nZVI. Earthworms of either species
215 were introduced to the soil after 30 d, and mortality, growth and reproduction measured after
216 2 and 4 weeks, as above (n=3).
217
218 **3. Results**

3.1. Effects of freshly added nZVI

Test criteria for the avoidance test were fulfilled: No earthworms died and their distribution between the two compartments was close to 50:50 in the control tests carried out with both the sandy soil and the OECD soil.

The effects of nZVI on the avoidance behavior of *E. fetida* and *L. rubellus* in sandy loam and OECD soil are shown in Figure 1A and 1B, respectively. For both species and both soils, worms tended to prefer soil amended with low concentrations of nZVI over the un-amended control. There was however no statistically significant avoidance by either worm species in either soil at nZVI concentrations ≤500 mg kg^{-1}. More than 60 % avoidance was observed at higher concentrations (≥750 mg nZVI kg $soil^{-1}$). ANOVA followed by a Tukey-Kramer HSD test also showed significant differences in avoidance between soils containing 1000 mg nZVI kg^{-1} and treatments with lower nZVI concentrations (up to 500 mg nZVI kg^{-1}). EC50 values calculated from the data on avoidance were 563 mg kg^{-1} for *E. fetida* and 532 mg kg^{-1} for *L. rubellus* in sandy loam soil, and 511 mg kg^{-1} for *E. fetida* and 582 mg kg^{-1} for *L. rubellus* in OECD soil (Table 1). EC50 values were not significantly different between species or soils at the 95 % confidence level.

The acute toxicity of nZVI to earthworms after 14 days in the two soils is shown in Table 2. There was no significant (p<0.05) effect of nZVI on mortality of either earthworm species at concentration ≤250 mg nZVI kg^{-1} for sandy loam soil and ≤625 mg nZVI kg^{-1} for OECD soil after 14 days exposure. At 500 mg nZVI kg^{-1}, 79 % and 89 % mortality was observed for *E. fetida* and *L. rubellus* in sandy loam soil, while no mortality was observed at the same concentration with the OECD soil. At 750 mg nZVI kg^{-1}, 100% mortality was observed for both species in sandy soil while in OECD soil 11 % mortality was observed for *L. rubellus*, and 44%. for *E. fetida* In OECD soil, 1000 mg nZVI kg^{-1} caused 100% mortality to *E. fetida*, and 89% for *L. rubellus*. After 28 days exposure, mortality also differed between

244 soils and earthworm species (Table 3). In sandy loam soil, 300 mg nZVI kg^{-1} caused 100 %
245 mortality for *E. fetida* and 89% mortality for *L. rubellus*. In OECD soil, the mortality was
246 67% and 22% at 500 mg nZVI kg^{-1} for *E. fetida* and *L. rubellus*, respectively. There was a
247 significant (p<0.05) difference in mortality between *E. fetida* and *L. rubellus* at 500 mg kg^{-1} in
248 OECD soil.

249 Test requirements for loss of body weight were fulfilled in 7 of 8 treatments, as loss of
250 body weight in control treatments remained below 15% (except one case where it was
251 15.2%), being the requirement limit (Spurgeon et al., 2003a; Spurgeon et al., 2003b). The
252 same authors also recommended that a weight loss >20% should be considered as an
253 indication of sub-lethal effects. At 200 mg nZVI kg^{-1}, both worms experienced significant
254 sub-lethal effects in sandy loam, while 300 mg nZVI kg^{-1} caused an even higher loss of body
255 weight in *L. rubellus* and resulted in lethal effects for *E. fetida* (Table. 4). A pilot experiment
256 had assessed effects at 100 mg nZVI kg^{-1} without observing any sub-lethal effects for either
257 species in sandy loam (results not shown). In OECD soil, at 250 mg nZVI kg^{-1}, *E. fetida* lost
258 20.2% of its body weight, while *L. rubellus* had a weight loss of <20%. Here, significant sub-
259 lethal effects were observed only at 500 mg nZVI kg^{-1} with about 50% weight loss in both
260 species.

261 Test validity criteria for the reproduction test were fulfilled in the control treatments
262 for both worm species with an average higher than three juveniles produced per adult
263 (Environmental Canada, 2007). All concentrations (100-1000 mg kg^{-1}) of freshly added nZVI
264 caused complete reproduction failure for both earthworm species and in both soils, with
265 neither cocoons nor juveniles being formed (results not shown).

266 **3.2. Effects of aged nZVI on earthworms**

Effects of aged nZVI on mortality of earthworms in the two soils after 14 and 28 days are shown in Table 5. Mortality showed the same pattern as in the treatment with freshly added nZVI. No mortality was observed for either earthworm species at concentrations ≤250 mg nZVI kg soil^{-1} with sandy loam soil and ≤500 mg nZVI kg soil^{-1} with OECD soil after 14 days. At 1000 mg nZVI kg^{-1}, 67 % mortality was observed for *L. rubellus* in OECD soil after 14 days, while the corresponding value for *E. fetida* was 78 %. All earthworms of both species exposed to 1000 mg aged nZVI kg^{-1} for 14 days died in the sandy loam soil. Likewise, after 28 days exposure to aged nZVI, the mortality differed between soils and earthworm species. In sandy loam soil, 250 mg nZVI kg^{-1} resulted in 22% mortality for both *E. fetida* and *L. rubellus*, while in the OECD soil the mortality was 78% and 0% at 500 mg nZVI kg^{-1} for *E. fetida* and *L. rubellus*, respectively. There was a significant difference (p<0.05) in mortality between *E. fetida* and *L. rubellus* at 750 mg nZVI kg^{-1} in OECD soil.

Effects on weight loss of earthworms were also observed after exposure to aged nZVI, but the relative decrease was lower than for freshly added nZVI (Table 4). Cocoon and juvenile production was severely affected in sandy loam soil, and no juveniles of either species were observed at any concentrations of nZVI (Table 6). In OECD soil, some cocoons were observed at 100 and 250 mg nZVI kg soil^{-1}. Soil pH and Eh varied only slightly between treatments, with pH ranging from 6.0 to 6.3 in sandy loam and from 6.3 to 7.3 in OECD soil. Eh varied from 50-99 mV in sandy loam and from -17 to 40 mV in OECD soil (the extremes were not associated with the highest or lowest nZVI concentration; results not shown).

4. Discussion

The use of nZVI to remediate polluted soils implies intentional spreading of engineered nano-particles into the environment, a practice that calls for assessment of possible hazards and risks. One of these risks concerns how soil biota is affected by high Fe concentrations, and

particularly high availability of reduced forms of Fe. The nZVI technology mainly targets treatment of pollutants in the subsoil under saturated conditions (Mueller et al., 2011). Still, mobility aspects are not resolved, meaning that nZVI may be transported to unsaturated soil inhabited by aerobic organisms, including earthworms.

Our results show that harmful effects of nZVI perceived as avoidance, loss of body mass and mortality of earthworms are only observed at high concentrations of nZVI (≥500 mg nZVI kg^{-1} soil), corresponding to concentrations likely to be found at or very close to the point of injection where earthworms are normally absent. Typically, nZVI is injected at a soil depth of 3-10 m in suspensions of 1-10 g nZVI l^{-1} (Li et al., 2006; Satapanajaru et al., 2008; He et al., 2010) and diluted by dispersion away from the injection hole in the order of decimeters to a few meters into the aquifer. Due to low mobility of nZVI (Saleh et al., 2007; Phenrat et al., 2009), predicted environmental concentrations of nZVI beyond a zone of treatment, and particularly in surface layers where earthworms are found, would be far lower than the EC50 values obtained for the endpoints above. A fourth endpoint tested in our experiments was reproduction, which turned out to be far more sensitive, with no complete reproduction observed at the lowest concentration tested (100 mg nZVI kg^{-1}). We assume that the cocoons we observed at the lowest exposure concentrations after nZVI aging were partially formed prior to the nZVI exposure, but that they never hatched due to residual effects of nZVI. The higher sensitivity of reproduction as an endpoint, and the failure in obtaining an EC50 for nZVI with respect to reproduction, calls for further studies. In this context it is interesting that even quite high concentrations of nZVI do not lead to a more pronounced avoidance, meaning that worms may enter zones where their capacity to reproduce ceases without perceiving the presence of nZVI as harmful. In other studies, avoidance by earthworms has been observed at lower concentrations than endpoints like weight loss and mortality, indicating that the stress and toxicity conferred by nZVI is different

317 from other metals/metalloids like As, Cu, Pb, Zn, (cf. Langdon et al., 2001; Langdon et al.,
318 2005; Lukkari and Haimi, 2005), and even Ag nanoparticles (Shoults-Wilson et al., 2011) or
319 pesticides like the pyrethroid insecticide cypermethrin (Zhou et al., 2008). One of the reasons
320 for this difference may be that iron is a common element in the soil and an essential element
321 required by all organisms at lower concentrations. When comparing nZVI to other metallic
322 ENMs, our results indicate that acute toxicity to earthworms is far lower than observed for
323 e.g. ENMs made from Cu (>65 mg kg^{-1}) or Ag (EC50: 60 mg kg^{-1}) (Lapied et al., 2010;
324 Unrine et al., 2010b; Lapied et al., unpublished results).

325 Soil texture and organic matter content strongly affect earthworm behavior (Curry and
326 Schmidt, 2006) and also the mobility and availability of nanoparticles in soil (Fang et al.,
327 2009; Wang et al., 2010). Toxicity of nanoparticles in soil will also be affected by soil
328 constituents like clay and organic matter (Navarro et al., 2008), and for nZVI we have
329 recently shown specifically that clay content affects toxicity to plants (El-Temsah and Joner,
330 2012). The OECD soil used in the present study contains 10% organic matter and 20% clay,
331 while the sandy loam soil we used had only 1% organic matter and 4% clay. The differences
332 in toxicity, where the OECD soil consistently gave the lowest toxicity and the highest
333 EC50/LD50-values, indicate that organic matter and/or clay also can reduce the toxicity to
334 earthworms. The contribution of each of these soil constituents could not be discerned in the
335 present study, as they were not varied individually. The effects of individual soil constituents
336 on the bioavailability of engineered nanoparticles have barely been assessed. Our own data on
337 sequential extraction of silver nanoparticles from different soils do however show that also
338 organic matter can reduce the availability of nanoparticles, as observed on both coated and
339 uncoated nanoparticles (Coutris et al. 2012).

340

341 **Effects of aged nZVI on earthworms**

342 A prominent feature of nZVI compared to other ENMs is that it oxidizes readily and
343 forms ferrous oxides that are already abundant in soils. This oxidation process is part of the
344 ageing that takes place upon contact with soil and its constituents, and is likely to result in a
345 corresponding reduction of toxicity to aerobic organisms. This spontaneous transformation
346 makes nZVI far less problematic as an anthropogenic input in natural systems compared to
347 other metal pollutants, including metallic ENMs. In comparison, non redox active metals use
348 decades to attain a significant reduction in bioavailability and consequent toxicity, as seen e.g.
349 for Cu when comparing spiked and 70 years-old contaminated soil (Scott-Fordsmand et al.,
350 2000).

351 We assumed that ageing of nZVI in soil would lead to partial oxidation of nZVI and
352 thus less reducing conditions and less adverse effects on earthworms. Indeed we observed that
353 nZVI aged for 30 days was less toxic to both worms than nZVI freshly added to soil. This is
354 in agreement with the findings of Phenrat et al. (2009) who observed that the oxidization of
355 nZVI in aged water converted into nontoxic magnetite and/or maghemite. Liu and Lowry
356 (2006) found that the half-life of nZVI in soil after injection was from 90-180 days, whereas
357 Kirschling et al. (2010) observed a total lifetime of nZVI added to three aquifer materials to
358 vary between 14 and 160 days, depending strongly on pH. Toxicity reduction upon partial
359 oxidation has been observed at relatively low nZVI concentrations (below 0.1 to 0.5 g L^{-1})
360 where a reduced toxic effect was observed on bacteria (*Escherichia coli*) (Li et al., 2010).
361 After complete oxidation (ageing for 250 d), an initial change in a microbial community
362 caused by nZVI was reversed, and lasting effects on metabolism and diversity of the
363 indigenous microorganisms could no longer be detected (Kirschling et al., 2010). In this case
364 a functional restoration thus occurred within a short time-span. Anaerobic soil bacteria are
365 probably the only indigenous organisms that will be exposed to nZVI during remediation
366 (Sevcu et al 2011), and ecotoxicity testing should include such organisms to describe the most

relevant biological impacts of this technology. To assess the over-all environmental impact, the degradation of chlorinated pollutants targeted by nZVI treatments should also be taken into account.

Organic matter and clay strongly affect not only the specific surface area of a soil, and thereby the surface area available for interactions with nanoparticles, but also influence soil aeration and oxygen diffusion. The differences we observed in toxicity of aged nZVI in the two contrasting soils are thus likely due to differences in soil composition. While clay minerals are known to reduce bioavailability and phytotoxicity of both inorganic (Lombi et al., 2002) and organic (Roberts et al., 2007) pollutants, organic matter is more important facilitating soil aeration. As for exposure to fresh nZVI, we were not able to distinguish which of the two parameters were decisive for the observed toxicity reduction of aged nZVI. Future experiments on ageing as a factor in toxicity of nZVI should feature different modes and degrees of aeration and assess the time-course of toxicity changes.

5. Conclusion

The present study shows, for the first time, that nZVI has potential negative effects on soil invertebrates. Acute toxicity was quite low, and related endpoints like growth depression and avoidance also had NOEC-values (no observed effect concentrations) between 200 and 500 mg nZVI kg^{-1}. Reproduction was affected at lower concentrations (<100 nZVI kg^{-1}) outside the tested range. We also observed that adverse effects were reduced with time, probably due to oxidation of Fe^0 during ageing of nZVI in soil who's surface was in contact with air. Further investigations should address ageing in a more detailed manner and distinguish the contribution of reducing conditions and high Fe uptake as factors causing adverse effects.

6. References

Auffan, M., Achouak, W., Rose, J., Roncato, M.A., Chaneac, C., Waite, D.T., Masion, A., Woicik, J.C., Wiesner, M.R., Bottero, J.Y., 2008. Relation between the redox state of iron-based nanoparticles and their cytotoxicity toward Escherichia coli. Environ Sci Technol 42, 6730-6735.

Capowiez, Y., Rault, M., Costagliola, G., Mazzia, C., 2005. Lethal and sublethal effects of imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). Biol Fertil Soils 41, 135-143.

Chang, M.C., Kang, H.Y., 2009. Remediation of pyrene-contaminated soil by synthesized nanoscale zero-valent iron particles. J Environ Sci Heal A 44, 576-582.

Chen, P.J., Su, C.H., Tseng, C.Y., Tan, S.W., Cheng, C.H., 2011. Toxicity assessments of nanoscale zerovalent iron and its oxidation products in medaka (*Oryzias latipes*) fish. Mar Pollut Bull 63, 339-346.

Conder, J.M., Lanno, R.P., Basta, N.T., 2001. Assessment of metal availability in smelter soil using earthworms and chemical extractions. J Environ Qual 30, 1231-1237.

Cook, S.M., 2009. Assessing the use and application of zero-valent iron nanoparticle technology for remediation at contaminated sites. US Environmental Protection Agency.

Coutris, C., Hertel-Aas, T., Lapied, E., Joner, E.J., Oughton, D.H., 2012. Bioavailability of cobalt and silver nanoparticles to the earthworm Eisenia fetida. Nanotoxicology 6, 186-195.

Coutris, C., Joner, E.J., Oughton, D.H., 2012. Aging and soil organic matter content affect the fate of silver nanoparticles in soil. Sci Total Environ 420, 327-333.

Cullen, L.G., Tilston, E.L., Mitchell, G.R., Collins, C.D., Shaw, L.J., 2011. Assessing the impact of nano- and micro-scale zerovalent iron particles on soil microbial activities: Particle reactivity interferes with assay conditions and interpretation of genuine microbial effects. Chemosphere 82, 1675-1682.

Curry, J.P., Schmidt, O., 2006. The feeding ecology of earthworms - A review. Pedobiologia 50, 463-477.

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms. Chapman and Hall, London.

El-Temsah, Y.S., Joner, E.J., 2012. Impact of Fe and Ag nanoparticles on seed germination and differences in bioavailability during exposure in aqueous suspension and soil. Environ Toxicol Chem 27, 42-49.

Elliott, D.W., Lien, H.L., Zhang, W.X., 2009. Degradation of Lindane by zero-valent iron nanoparticles. J Environ Eng-ASCE 135, 317-324.

Environmental Canada, 2007. Biological test methods for measuring the toxicity of contaminated soil to earthworms (*Eisenia andrei*, *Eisenia fetida* or *Lumbricus terrestris*) In: Environmental Protection Series, Biological Test Methods. Method Development and Applications Section. Environmental Technology Centre, Ottawa.

Fang, J., Shan, X.Q., Wen, B., Lin, J.M., Owens, G., 2009. Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns. Environ Pollut 157, 1101-1109.

He, F., Zhao, D.Y., Paul, C., 2010. Field assessment of carboxymethyl cellulose stabilized iron nanoparticles for in situ destruction of chlorinated solvents in source zones. Water Res 44, 2360-2370.

Hund-Rinke, K., Wiechering, H., 2001. Earthworm avoidance test for soil assessments - an alternative for acute and reproduction tests. J Soil Sediments 1, 15–20.

ISO, 2005. Draft ISO-17512: Soil quality and avoidance test for evaluating the quality of soils and the toxicity of chemicals. Test with earthworms (*Eisenia fetida/andrei*) International Organization for Standardization, Geneva, Switzerland.

Karn, B., Kuiken, T., Otto, M., 2009. Nanotechnology and in situ remediation: A review of the benefits and potential risks. Environ Health Persp 117, 1823-1831.

Keane, E., 2009. Fate, transport, and toxicity of nanoscale zero-valent iron (nZVI) used during superfund remediation. U.S. Environmental Protection Agency.

Kirschling, T.L., Gregory, K.B., Minkley, E.G., Lowry, G.V., Tilton, R.D., 2010. Impact of nanoscale zero valent iron on geochemistry and microbial populations in trichloroethylene contaminated aquifer materials. Environ Sci Technol 44, 3474-3480.

Langdon, C.J., Hodson, M.E., Arnold, R.E., Black, S., 2005. Survival, Pb-uptake and behaviour of three species of earthworm in Pb treated soils determined using an OECD-style toxicity test and a soil avoidance test. Environ Pollut 138, 368-375.

Langdon, C.J., Piearce, T.G., Meharg, A.A., Semple, K.T., 2001. Survival and behaviour of the earthworms *Lumbricus rubellus* and *Dendrodrilus rubidus* from arsenate-contaminated and non-contaminated sites. Soil Biol Biochem 33, 1239-1244.

Lanno, R., Wells, J., Conder, J., Bradham, K., Basta, N., 2004. The bioavailability of chemicals in soil for earthworms. Ecotox Environ Safe 57, 39-47.

Lapied, E., Moudilou, E., Exbrayat, J.-M., Oughton, D.H., Joner, E.J., 2010. Silver nanoparticle exposure causes apoptotic response in the earthworm *Lumbricus terrestris* (Oligochaeta). Nanomedicine 5, 975-984.

Lee, C., Kim, J.Y., Lee, W.I., Nelson, K.L., Yoon, J., Sedlak, D.L., 2008. Bactericidal effect of zero-valent iron nanoparticles on *Escherichia coli*. Environ Sci Technol 42, 4927-4933.

Li, X.Q., Elliott, D.W., Zhang, W.X., 2006. Zero-valent iron nanoparticles for abatement of environmental pollutants: Materials and engineering aspects. Crit Rev Solid State 31, 111-122.

Li, Z., Greden, K., Alvarez , P.J.J., Gregory, K.B., Lowry, G.V., 2010. Adsorbed polymer and NOM limits adhesion and toxicity of nano scale zerovalent iron to *E. coli*. Environ Sci Technol 44, 3462-3467.

Liu, Y.Q., Lowry, G.V., 2006. Effect of particle age (Fe-0 content) and solution pH on NZVI reactivity: H-2 evolution and TCE dechlorination. Environ Sci Technol 40, 6085-6090.

Lombi, E., Zhao, F.J., Wieshammer, G., Zhang, G.Y., McGrath, S.P., 2002. *In situ* fixation of metals in soils using bauxite residue: Biological effects. Environ Pollut 118, 445-452.

Lukkari, T., Haimi, J., 2005. Avoidance of Cu- and Zn-contaminated soil by three ecologically different earthworm species. Ecotox Environ Safe 62, 35-41.

Ma, W.-C., 2005. Critical body residues (CBRs) for ecotoxicological soil quality assessment: copper in earthworms. Soil Biol Biochem 37, 561-568.

Marques, C., Pereira, R., Goncalves, F., 2009. Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. J Soil Sediment 9, 137-147.

Mueller, N.C., Braun, J., Bruns, J., Černík, M., Rissing, P., Rickerby, D., Nowack, B., 2011. Application of nanoscale zero valent iron (NZVI) for groundwater remediation in Europe. Environ Sci Pollut Res.

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.-J., Quigg, A., Santschi, P.H., Sigg, L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17, 372–386.

OECD, 1984. Guideline for the Testing of Chemicals: Earthworm Acute Toxicity Tests, TG 207. Organization for Economic Cooperation and Development, Paris.

Park, H., Park, Y.M., Oh, S.K., You, K.M., Lee, S.H., 2009. Enhanced reduction of nitrate by supported nanoscale zero-valent iron prepared in ethanol-water solution. Environ Technol 30, 261-267.

Phenrat, T., Long, T.C., Lowry, G.V., Veronesi, B., 2009. Partial oxidation ("aging") and surface modification decrease the toxicity of nanosized verovalent iron. Environ Sci Technol 43, 195-200.

Roberts, M.G., Rugh, C.L., Li, H., Teppen, B.J., Boyd, S.A., 2007. Reducing bioavailability and phytotoxicity of 2,4-dinitrotoluene by sorption on K-smectite clay. Environ Toxicol Chem 26, 358-360.

Rombke, J., Jansch, S., Didden, W., 2005. The use of earthworms in ecological soil classification and assessment concepts. Ecotox Environ Safe 62, 249-265.

Saleh, N., Sirk, K., Liu, Y.Q., Phenrat, T., Dufour, B., Matyjaszewski, K., Tilton, R.D., Lowry, G.V., 2007. Surface modifications enhance nanoiron transport and NAPL targeting in saturated porous media. Environ Engin Sci 24, 45-57.

Satapanajaru, T., Anurakpongsatorn, P., Pengthamkeerati, P., Boparai, H., 2008. Remediation of atrazine-contaminated soil and water by nano zerovalent iron. Water Air Soil Poll 192, 349-359.

Schinner, F., Öhlinger, R., Kandeler, E., Margesin, R., 1996. Methods in Soil Biology. Springer, London.

Scott-Fordsmand, J.J., Weeks, J.M., Hopkin, S.P., 2000. Importance of contamination history for understanding toxicity of copper to earthworm *Eisenia fetida* (*Oligochaeta: Annelida*), using neutral-red retention assay. Environ Toxicol Chem 19, 1774-1780.

Ševců, A., El-Temsah, Y.S., Joner, E.J., Černík, M., 2011. Oxidative stress induced in microorganisms by zero-valent iron nanoparticles. Microb Environ 26, 271–281.

Shoults-Wilson, W.A., Zhurbich, O.I., McNear, D.H., Tsyusko, O.V., Bertsch, P.M., Unrine, J.M., 2011. Evidence for avoidance of Ag nanoparticles by earthworms (*Eisenia fetida*). Ecotoxicology 20, 385-396.

Spurgeon, D.J., Svendsen, C., Weeks, J.M., Hankard, P.K., Stubberud, H.E., Kammenga, J.E., 2003a. Quantifying copper and cadmium impacts on intrinsic rate of population increase in the terrestrial oligochaete Lumbricus rubellus. Environ Toxicol Chem 22, 1465-1472.

Spurgeon, D.J., Weeks, J.M., Van Gestel, C.A.M., 2003b. A summary of eleven years progress in earthworm ecotoxicology. Pedobiologia 47, 588-606.

Unrine, J.M., Hunyadi, S.E., Tsyusko, O.V., Rao, W., Shoults-Wilson, W.A., Bertsch, P.M., 2010a. Evidence for bioavailability of Au nanoparticles from soil and biodistribution within earthworms (*Eisenia fetida*). Environ Sci Technol 44, 8308-8313.

Unrine, J.M., Tsyusko, O.V., Hunyadi, S.E., Judy, J.D., Bertsch, P.M., 2010b. Effects of particle size on chemical speciation and bioavailability of copper to earthworms (*Eisenia fetida*) exposed to copper nanoparticles. J Environ Qual 39, 1942-1953.

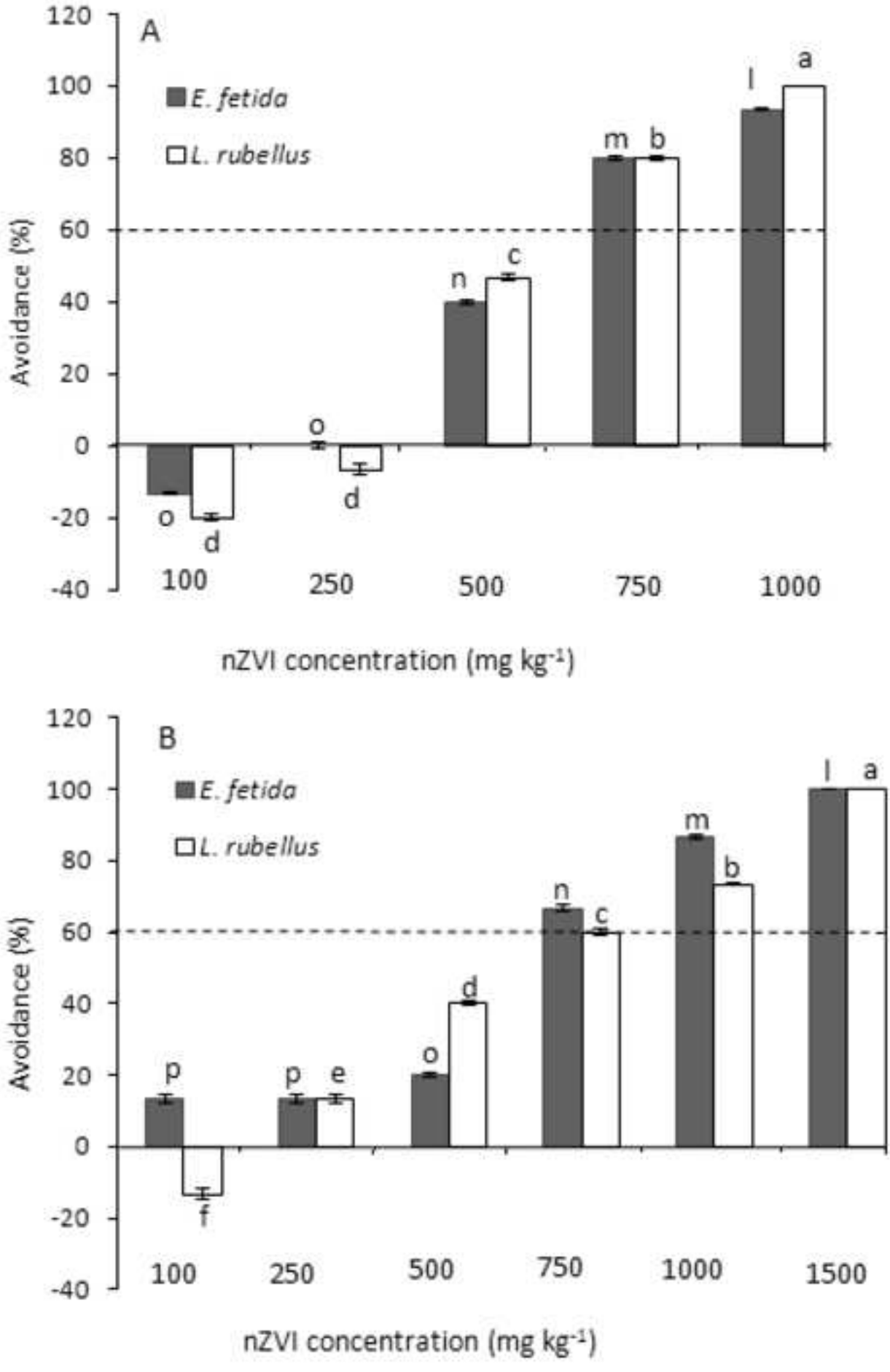
Wang, Y., Li, Y., Kim, H., Walker, S.L., Abriola, L.M., Pennell, K.D., 2010. Transport and retention of fullerene nanoparticles in natural soils. J Environ Qual 39, 1925-1933.

Zhang, W.X., 2003. Nanoscale iron particles for environmental remediation: An overview. J Nanopart Res 5, 323-332.

Zhou, S.P., Duan, C.Q., Wang, X.H., Michelle, W.H.G., Yu, Z.F., Fu, H., 2008. Assessing cypermethrin-contaminated soil with three different earthworm test methods. J Environ Sci-China 20, 1381-1385.

Figure

Click here to download high resolution image



Table

Click here to download Table: El-Temsah Revised Tables.docx

Table 1. EC_{50} for avoidance after 48 h and LC_{50} for acute toxicity (14 d exposure) and 95% confidence limits.

Species	Soil type	EC_{50}	Lower	Upper
L.rubellus	OECD	582	450	726
	Sandy loam	532	502	565
E. fetida	OECD	511	83.2	1184
	Sandy loam	563	527	597
		LC_{50}		
L.rubellus	OECD	866	842	890
	Sandy loam	447	438	457
E. fetida	OECD	-	-	-
	Sandy loam	399	337	460

Table 2. Acute toxicity (mortality after 14 d exposure) to a range of concentrations of nZVI towards *E. fetida* and *L. rubellus* living in sandy loam or OECD soil (n= 3; ± SEM).

nZVI (mg kg soil^{-1})	Mortality (%)			
	E. fetida		*L. rubellus*	
	Sandy loam	OECD soil	Sandy loam	OECD soil
0	0	0	0	0
100	0	ND	0	ND
200	0	ND	0	ND
250	ND	0	ND	0
300	33±0	ND	0	ND
400	33±23.6	ND	11±13.6	ND
500	79±13.6	0	89±14	0
625	90±13.6	0	100	0
750	100	44	100	11±13.6
1000	100	100	100	89±13.6

ND= not determined

Table 3. Chronic toxicity (mortality after 28 d exposure) to a range of concentrations of nZVI towards *E. fetida* and *L. rubellus* in sandy loam or OECD soil (n= 3; ± SEM).

nZVI (mg kg soil^{-1})	Mortality (%)			
	E. fetida		*L. rubellus*	
	Sandy loam	OECD soil	Sandy loam	OECD soil
0	0	0	0	0
100	0	ND	0	ND
200	56±27.2	ND	22±13.5	ND
250	ND	0	ND	0
300	100	ND	89±13.6	ND
400	100	ND	100	ND
500	100	67±23.6	100	22±13.5
625	100	78±13.6	100	22±13.5
750	100	100	100	44±27.2
1000	100	100	100	100

ND= not determined

Table 4. Percent change in body weight of earthworms during 28 d exposure at a range of concentrations of freshly added and aged (30d) nZVI in sandy loam or OECD soil. Negative values indicate weight loss (n= 3; ± SEM).

nZVI	Freshly added nZVI, sandy loam		Aged nZVI, sandy loam	
(mg kg soil^{-1})	*E. fetida*	*L. rubellus*	*E. fetida*	*L. rubellus*
0	13.8±2.1	4.9±8.6	-15.2±8.2	0
100	ND	ND	3.8±12.3	-5.3±9.2
200	-65.5±10.6	-42.7±16.6	ND	ND
250	ND	ND	-48.7±10.3	-47.2±9.4
300	*	-76.1±9.2	*	*
400	*	*	*	*
	Freshly added nZVI, OECD soil		Aged nZVI, OECD soil	
0	16.4±6.2	25.5±10.4	4.3±5.4	11.7±9.8
100	ND	ND	7.5±10.2	7.8±6.5
250	-20.2±3.5	-10.4±5.6	-17.4±7	-3.6±9.8
500	-42.4±4.4	-34±17.5	-51.8±6.7	-41.7±7.5
625	-45.3±12.7	-36.4±5.5	ND	ND
750	*	-62.6±10.2	*	-54.7±8.5
1000	*	*	*	*

* No worms survived

Table 5. Acute and chronic toxicity (mortality after 14 and 28 d exposure) to a range of concentrations of aged (30 d) nZVI to *E. fetida* and *L. rubellus* living in sandy loam or OECD soil (n= 3; ± SEM).

nZVI (mg kg soil^{-1})	Mortality (%) after 14 d			
	E. fetida		*L. rubellus*	
	Sandy loam	OECD soil	Sandy loam	OECD soil
0	0	0	0	0
100	0	0	0	0
250	0	0	0	0
500	67±40.8	0	100	0
750	100	67±23.6	100	0
1000	100	78±27.1	100	67±0
2000	100	100	100	100
	Mortality (%) after 28 d			
0	0	0	0	0
100	0	0	0	0
250	22±13.6	0	22±13.6	0
500	100	78±27.1	100	0
750	100	100	100	33±23.6
1000	100	100	100	100
2000	100	100	100	100

Table 6. Effects of aged (30 d) nZVI on reproduction of *E. fetida* and *L. rubellus* (number of cocoons and juveniles produced during 28 d exposure) (n= 3; ± SEM).

nZVI (mg kg^{-1})	Sandy loam			
	E. fetida		*L. rubellus*	
	Cocoons	Juveniles	Cocoons	Juveniles
0	13. 7±2	12.3±1.1	9.7±1.5	26±1.2
100	0	0	2	0
250	0	0	0	0
500	0	0	0	0
750	0	0	0	0
1000	0	0	0	0
	OECD soil			
0	12.3±3.3	15.3±2	19.3±1.8	27.3±3.1
100	11.7±1.1	0	6.7±1.1	0
250	5±0.7	0	2.3±0.4	0
500	0	0	0	0
750	0	0	0	0
1000	0	0	0	0