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Amendment application in a multi-contaminated mine soil: Effects on soil enzymatic activities and ecotoxicological characteristics.

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Abstract

Several amendments were tested on soils obtained from an arsenopyrite mine, further planted with *Arrhenatherum elatius* and *Festuca curvifolia*, in order to assess their ability to improve soil’s ecotoxicological characteristics. The properties used to assess the effects were: soil enzymatic activities (dehydrogenase, β-glucosidase, acid phosphatase, urease, protease and cellulase), terrestrial bioassays (*Eisenia fetida* mortality and avoidance behavior), and aquatic bioassays using a soil leachate (*Daphnia magna* immobilisation and *Vibrio fischeri* bioluminescence inhibition).

The treatment with FeSO₄ 1% w/w was able to reduce extractable As in soil, but increased the extractable Cu, Mn and Zn concentrations, as a consequence of the decrease in soil pH, in relation to the unamended soil, from 5.0 to 3.4, respectively. As a consequence, this treatment had a detrimental effect in some of the soil enzymatic activities (e.g. dehydrogenase, acid phosphatase, urease and cellulase), did not allow plant growth, induced *E. fetida* mortality in the highest concentration tested (100% w/w), and its soil leachate was very toxic towards *D. magna* and *V. fischeri*. The combined application of FeSO₄ 1% w/w with other treatments (e.g. CaCO₃ 1% w/w and paper mill 1% w/w) allowed a decrease in extractable As and metals, and a soil pH
value closer to neutrality. As a consequence, dehydrogenase activity, plant growth and some of the bioassays identified those as better soil treatments to this type of multi-contaminated soil.

**Keywords**

Multi-contaminated soil, Amendments, Soil remediation, Enzymatic activities, Bioassays, Ecotoxicity

**1 Introduction**

Soil health has been defined as the capacity of a soil to function as a vital living system within ecosystem and land-use boundaries, sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health (Doran and Zeiss 2000). Awareness concerning the importance of the soil functions’ recovery after the occurrence of a disturbance has risen since the 90’s. Analyses only on chemical basis do not provide evidence for toxicological consequences in biota (Fent, 2004), as chemical tests do not reflect the effect of pollutants on organisms and the interactions among contaminants, matrix and biota (ISO/DIS 17402, 2006). They provide weak information about the bioavailability or toxicity of contaminants and are insufficient to offer an insight into the potential ecological risk. On the contrary, ecotoxicity tests provide an integrated measure of bioavailability and detrimental effects of contaminants in the ecosystem (Alvarenga et al. 2007). Bioassays using ecosystem’s representative organisms are reported to be valuable tools to evaluate potential ecological risks in disturbed soils (Van Gestel et al. 2001; Leitgib et al. 2007).
Sublethal avoidance behaviour tests with *Eisenia fetida* after a mortality assay have been proposed in this work. Avoidance tests are used to assess the ecological risk in lower tier levels (Natal da Luz et al. 2004), and are appropriate to evaluate the habitat function of soil and the influence of contaminants on earthworms behaviour (ISO/DIS 17512-1, 2008). According to Amorim et al. (2008), avoidance tests with earthworms are a simple and ecologically relevant measurable endpoint for assessing the effect of metals in soil on earthworm movement. Earthworm presence in soil is very common and represents 60-80% of the total soil animal/invertebrate biomass. Furthermore, *E. fetida* can be reproduced easy and quickly in the laboratory and its sensitivity to contaminants are in the same range than other more important earthworms species in soils. Their locomotor abilities enable them to avoid unfavourable environments (Filser et al. 2000) and the behaviour parameter was equally or more sensitive than other sublethal parameters as reproduction or growth (Loureiro et al. 2005).

Bioassays using the freshwater crustacean *Daphnia magna* and the marine luminescent bacterium *Vibrio fischeri* were developed to test toxicity in aquatic environments. However, procedures can be adapted to soils, using leachates extracted with water, in order to assess the impact of soil composition on ground water (Loureiro et al. 2005; Alvarenga et al. 2008a). *Vibrio fischeri* bioassays have demonstrated to be sensitive to heavy metals and can detect acute and sublethal effects caused by a large number of chemicals (Ribo and Kaiser, 1983). The tests determine the decrease of the luminescence emitted by the bacteria *V. fischeri* when exposed to contaminants (Martín et al, 2010). The luminescence intensity can elucidate the metabolic bacteria status (Wolska et al. 2007), being the reduction of light proportional to the toxic effect produced by the substance tested (Mortimer et al. 2008). Also, *D. magna* immobilisation (acute tests) and reproduction (chronic tests) have been successfully
used by other authors as indicators of contamination by heavy metals, and other contaminants, on different matrixes (Renoux et al. 2001; Kungolos et al. 2004; Alvarenga et al. 2007, 2009; Wilke et al. 2008), as these invertebrates are identified as being quite sensitive to a wide range of contaminants (Bostan et al. 2005; Barata et al. 2007; Miranda et al. 2010).

Enzyme activities have also been proposed as potential indicators of soil quality and degradation (Pérez de Mora et al. 2006) and their analysis have been recommended in order to evaluate risks associated with trace elements (Hinojosa et al. 2004, 2008). The adverse effects of arsenic and heavy metals on the biological functions of soils, such as soil diversity and microbial activity, have been observed (Chander et al. 2001, Renella et al. 2008). Modifications of microbial community due to environmental factors should be reflected on the level of soil enzymatic activities (Kandeler et al. 1996). Also, it has been demonstrated that soil enzymatic activities have a rapid response to changes in soil management (Madejón et al. 2009). For example, they provide information about inappropriate agronomical practices which lead to reductions in the activities of some enzymes (Pérez de Mora et al. 2005).

In this study, several amendments have been selected to elucidate whether their addition to soils contaminated with arsenopyrite residues improve the soil biological activity. The amendments were chosen according to their effectiveness in reducing arsenic and metals, as iron-bearing compounds, liming materials and combinations of both (Hartley et al. 2004; Moreno-Jiménez et al. 2012). Calcium carbonate and calcium oxide-bearing materials are commonly incorporated into soils for remediation purposes (Madejón et al. 2002). Among them, paper mill, a by-product from de-inking of paper, contains a considerable proportion of calcium carbonate and its addition has been shown to diminish the mobile fractions of metals in soils (Gadepalle et al. 2007;
Méndez et al. 2009). Furthermore, its recycling constitutes a very valuable alternative to minimise its land disposal.

Bioassays can provide information about the evolution of disturbed soils by the incorporation of amendments, the sustainability of agricultural practices and the improvement in soil quality. As the sensitivity of the organisms mentioned above to toxicants has been proved, changes in their response are expected to occur with the addition of ameliorants to the soils.

The aim of this work was to evaluate the improvement in the ‘health’ of soils contaminated with arsenic and heavy metals after the addition of amendments in comparison with a situation in which no amendments were added. For this purpose, all the biological parameters mentioned have been used: soil enzymatic activities (dehydrogenase, β-glucosidase, acid phosphatase, urease, protease and cellulase), terrestrial bioassays (Eisenia fetida mortality and avoidance behavior), and aquatic bioassays using a soil leachate (Daphnia magna immobilisation and Vibrio fischeri bioluminescence inhibition).

2 MATERIALS AND METHODS

2.1 Experimental set-up

Soil was collected in the surroundings of an ancient arsenopyrite mine located in Bustarviejo, NW Madrid (Spain). The main minerals present in the tailing dumps are arsenopyrite and matildite. Matildite is a sulphide mineral containing bismuth and silver and appears in the form of small grains associated with arsenopyrite. Silver was
extracted from the site from the 17th century to the 1980s. The mine is in a high valley with two streams passing through it. Tailing dumps were established on the slopes above the stream banks, using the natural depression of the two rivers, so contamination may reach water bodies through soil erosion and contamination dispersion. Further information about the description of the mine location and the contamination dispersion across the area can be found in Moreno-Jiménez et al. (2009, 2010).

Contaminated material was collected from the 10 upper cm from several points in the dumping tailings, and the uncontaminated bulk soil from the surroundings of the mine (20 upper cm of the soil). Both materials were homogenised separately and after that they were air-dried for one week and sieved to 4 mm. Uncontaminated soil plus dumping material were mixed in a 60:40 proportion (w/w) by hand in order to obtain a homogenised substrate for plant growth, and the mixture was added to the pots (2 kg in 2-L pots). This proportion was selected to represent the level of soil pollution found in the soils surrounding the mine but with minimum physico-chemical characteristics to support plant development. The amendments were added to the mixture before pot filling, and they were (the abbreviations used for each treatment along this work are shown between brackets):

- No amended (‘NA’)
- FeSO₄ 1% w/w (‘Fe’)
- CaCO₃ 1% w/w (‘Ca’)
- FeSO₄ 1% w/w + CaCO₃ 1% w/w (‘Fe+Ca’)
- Paper mill 1% w/w (‘PM’)
- FeSO₄ 1% w/w + paper mill 1% w/w (‘Fe+PM’)
FeSO₄ and CaCO₃ were obtained from Sigma Aldrich and paper mill was supplied by Holmen Paper (Madrid, Spain). Pots were left to equilibrate for one month at 70% water holding capacity (previously determined). After one month, pots were divided in two sections. 100 seeds of *Arrhenatherum elatius* subsp. Bulbosum and 100 seeds of *Festuca curvifolia* coming from plants growing in the mine were sown in each half of the pot, allowing them to grow for five weeks. The experiment was carried out in a greenhouse under controlled conditions (10-28 °C and 60-80% relative humidity). A randomised design with four replicates for each treatment was used.

At the end of the experiment, plant shoots were cut and their fresh weight was recorded. Plant material was washed thoroughly with tap and deionised water, dried at 60 °C during three days, and milled for the mineral analysis. Pots were emptied and soil was homogenised by hand. A portion of the soil was kept refrigerated (4 °C) at their field moisture to be used in the determination of the soil enzymatic activities. The remaining soil was air-dried and sieved to 2 mm for analytical determinations and ecotoxicological evaluation.

2.2 Soil and plant analytical characterization

An initial characterisation of the uncontaminated soil, dumping material, the composite mixture of the two latter materials (60:40 proportion w/w), and paper mill was performed (Table 1). Soil pH measurement was determined in a 1:2.5 (w/v) soil to deionised water suspension shaken for 30 min, with an electrode Crison 5014. Electrical conductivity was determined in a 1:5 (w/v) soil to deionised water suspension shaken for 1 h, with an electrode Crison CE5070 (MAPA 1994). The same electrodes were used to measure pH and electrical conductivity of the paper mill, but using a 1:10 (w/v)
paper mill to deionised water suspension shaken for 1 h. Organic matter content (OM) was analysed by loss-on-ignition, at 550 °C for 5 hours in a muffle furnace. Pseudo-total metal (Cu, Mn, Zn and Cd) and As concentrations were extracted by means of an HNO$_3$:H$_2$O$_2$ digestion in autoclave (Wenzel et al. 2001). The extractable concentrations of the same elements in those matrixes, were determined by shaking a sub-sample with 0.1 M (NH$_4$)$_2$SO$_4$, in a soil-to-solution ratio 1:10 (w/v) during 4 hours. The suspension was filtered and As and metals concentrations were analysed in the filtrate. Arsenic was analysed by atomic fluorescence spectroscopy (Milenium Excalibur PS Analytical) and metals by atomic absorption spectroscopy, using a Varian apparatus (Perkin Elmer AAnalyst 800). At the end of the experiment, soil pH, OM content, pseudo-total and extractable As and metals were also determined using the same procedures (Table 2). The effect of the amendments application on soil available P and K was also assessed, using the Egner–Riehm method in their analytical determination (Riehm, 1958).

Soil leachates were extracted according to DIN 38 414-S4 (1984). Soils (dried and sieved to 2 mm) were shaken under constant agitation for 24 h at room temperature in a soil-to-liquid ratio 1:10 (w/v) with deionised water. The leachates were separated by centrifugation and filtrated through a membrane filter (pore size 0.45 µm). Leachates were further analysed for pH, metals and As, using the above mentioned methodologies.

2.3 Soil enzymatic activities

Soil enzymatic activities (dehydrogenase, β-glucosidase, acid phosphatase, urease, protease and cellulase) were analysed in the refrigerated soil. Before analysis, soil samples were passed through a 2 mm sieve, and their dry matter content was
determined by oven-drying the soil at 105 °C for 48 hours until constant weight to express the enzymatic activity on dry weight basis.

To measure dehydrogenase activity, soils were incubated with 2,3,5-triphenyltetrazolium chloride (16 h, 25 °C, darkness) which was reduced to triphenylformazan. This last molecule was determined spectrophotometrically at 546 nm (Tabatabai 1994). β-glucosidase was determined according to Eivazi and Tabatabai (1988) as described by Alef and Nannipieri (1995a): 1 g of soil was incubated with p-nitrophenyl-β-glucopyranoside during 1 hour at 37 °C. The resultant end product, p-nitrophenol, was determined spectrophotometrically at 400 nm. Acid phosphatase was determined according to Eivazi and Tabatabai (1977) as described by Alef et al. (1995): 1 g of soil was incubated with p-nitrophenylphosphate during 1 hour at 37 °C. The resultant product, p-nitrophenol, was determined spectrophotometrically at 400 nm. Urease activity was analysed according to Kandeler and Gerber (1988), as described in Alef and Nannipieri (1995b): 5 g of soil were incubated with urea for 2 hours at 37 °C. The nitrogen (N-NH₄⁺) released was extracted with Na-salicilate/NaOH and Na-dicloroisonianyde and determined spectrophotometrically at 690 nm. Protease activity was determined spectrophotometrically (700 nm) after the incubation of the soil with caseynate during 2 h at 50 °C as according to the method developed by Ladd and Butler (1972) and described in Alef and Nannipieri (1995c). Cellulase activity was determined according to Hope and Burns (1987). Cellulases are enzyme systems that degrade cellulose and release reducing sugars as the end product. The term refers to the combined action of endo-1,4-β-D-glucanase (EC 3.2.1.4), exo- 1,4- β-D-glucanase (EC 3.2.1.91) and β-D-glucosidase (EC 3.2.1.21) on Avicel®, a purified depolymerised alpha cellulose. The released reducing sugars were determined spectrophotometrically at 520 nm.
2.4 Ecotoxicity bioassays

Soil ecotoxicity evaluation was performed using terrestrial tests, to assess the improvement in soil habitat function, and aquatic tests, to assess the improvement in soil retention function. The terrestrial tests (direct tests) comprised the *Eisenia fetida* mortality and avoidance behavior. Two aquatic species were used in the aquatic tests (indirect exposure) using the soil leachate: immobilisation of *D. magna* and luminescence inhibition of *V. fischeri*. These bioassays were carried out using soil leachates, extracted according to DIN 38 414-S4 (1984), obtained and analysed as described above.

2.4.1 *Eisenia fetida* mortality and avoidance behaviour bioassays

The *E. fetida* mortality bioassay was carried out following the OECD procedure (OECD 207 1984). The tests were performed using a soil composite sample, prepared by mixing four subsamples from each pot per treatment. Then the composite soil sample was diluted with the OECD artificial soil (10% peat, 20% clay and 70% quartz sand, pH 6-7 adjusted with calcium carbonate), which was also used as the control (dilutions were 100, 50, 25, 12.5% w/w test soil). Moisture was adjusted in order to reach 60% of the mixtures water holding capacity. The equivalent to 400 g of the mixture dry matter were introduced in plastic containers (approximately 1 L capacity, 190-111 mm), four replicates per concentration, and ten *E. fetida* earthworms (each organism weighing 0.2-0.4 g) were placed into the containers, sealed and stored in an acclimatized room at 20±2 °C in continuous light (to ensure that worms remained in the test medium.
throughout the duration of the test). After 14 days, mortality was registered and, whenever possible, EC\textsubscript{20} and EC\textsubscript{50} (soil concentration, \% w/w, at which a toxic effect on 20 or 50\% of the exposed organisms can be observed) were determined.

Avoidance behaviour tests were carried out according to ISO/DIS 17512-1 (2008). After mortality tests, avoidance bioassays were performed with the treatments and their respective dilutions where \textit{E. fetida} organisms survived after 14 days. The assay evaluates the tendency that earthworms have to avoid the test soil whilst preferring the control soil. Two-section containers with two identical sides were divided by means of a separator (approximately 1 L capacity, 190-111 mm). Soil and their dilutions were prepared as in the mortality tests. One half of the container was filled with artificial soil, used as a control, and the other half with the test soil or their respective dilutions. The separator was removed and ten \textit{E. fetida} earthworms were placed onto the separating line of each test vessel. Containers were placed in an acclimatized room at 20±2 \textdegree C in continuous light. The test was run with four replicates. After 48 hours, control and test soils were separated by inserting the divider and the number of worms was determined in each section of the containers. Animals that were located on the separation line were considered as being in the soil to which the animal’s head was directed.

The assessment of the ‘soil habitat’ function was performed as proposed in the method (ISO/DIS 17512-1 2008): the soil is considered to have a limited ‘habitat function’ if more than 80\% of worms are found in the control soil, indicating that there has been an impact in their behaviour.

The calculations made to determine whether the habitat function has been impaired used the following formulae:
\[ \%AV = \left( \frac{(C - T)}{N} \right) \cdot 100 \]

Where C is the number of worms found in the artificial soil (control); T the number of worms found in the test soil and N the total number of worms in the vessel. The percentage of avoidance considering 100 earthworms, 20 of which are found in the test soil and the rest in the artificial soil would have a value of \( AV(\%) = \left( \frac{80 - 20}{100} \right) \cdot 100 = 60 \). So, avoidance percentages above 60 mean that less than 20% of the organisms are in the test soil and its habitat function is impaired.

2.4.2 *Daphnia magna* immobilisation

The *D. magna* acute immobilisation test was performed according to the standardised method ISO 6341 (1996). Soil leachates were diluted with holding and dilution water prepared according to ISO 6341 (1996), to obtain the concentrations 50, 25, 12.5, 6.25% (v/v). Furthermore, soil leachates without being diluted (100%) were tested, and holding and dilution water was used as a control (four replicates per concentration). Five young daphnids, aging less than 24 hours at the start of the test, were exposed to 25 mL of the test solution, for 48 h in a conditioned chamber with 16 h light/ 8 h dark cycle and 20±2 °C. After 48 h, immobilisation was recorded. EC_{20} and EC_{50} were calculated whenever possible.

2.4.3 Inhibition of light emission of *Vibrio fischeri*

Inhibition of the luminescence of *V. fischeri* (NRRL B-11177) was performed according to the standardised method ISO 11348-2 (1998). Similar soil dilutions as in *D.*
*magna* were used against a control (NaCl 2\% w/v). The decrease of light emission was tested 15 and 30 min after the bacteria solution was in contact with the sample to be tested. The equipment used was LUMISStox 300. Measures were carried out in duplicate. EC<sub>20</sub> and EC<sub>50</sub> were calculated whenever possible.

2.5 Statistical analyses of data

Differences between means were tested using the statistical program SPSS 15.0. Statistical tests performed include one-way ANOVA followed by Duncan’s test to determine whether there are any significant differences between the means of each treatment, assuming a normally distribution of the dependent variable data and homogeneity of variances. Kruskal-Wallis tests alongside with Mann-Whitney post hoc tests were performed when the assumption of normality was rejected. Robust tests of equality of means (Welch or Brown-Forsythe tests followed by Games-Howell post hoc tests) were run when homogeneity of variances assumptions was violated and the use of the one-way ANOVA was inappropriate.

EC<sub>20</sub> and EC<sub>50</sub> of *D. magna* immobilisation bioassays were calculated with the statistical program Minitab 15.0. Values of EC<sub>20</sub> and EC<sub>50</sub> in the *V. fischeri* bioluminescence inhibition were determined using the software of the equipment LUMISStox 300, from Lange®. *Eisenia fetida* avoidance tests percentages were treated with the Fischer exact test. This test compares the distribution of animals in relation to an expected distribution assuming the non-existence of an avoidance response in a soil (Zar 1999).

Principal component analysis (PCA) was performed using SPSS 15.0. PCA provides information about the most meaningful parameters which describe the whole
data set. It is a statistical tool which reduces a larger set of variables into a smaller set called principal components, which are linear combinations of the original ones and explain most of the variance in the original variables (Jolliffe 2005). The type of rotation used was ‘Varimax’ approach, which provides an orthogonal solution.

3 Results and discussion

3.1 Effect of amendments on soil characteristics, plant growth and soil enzymatic activities

Treatments had neither effect on pseudo-total concentrations of As, Cu, Mn, Zn, and Cd nor on soil OM content (Table 2). However, there were significant differences among treatments on some of the soil properties, mainly on their soil pH, extractable P and K, and the content of extractable As, Cu, Mn and Zn (Table 2). The incorporation of FeSO₄ to the soils (treatment ‘Fe’ ) reduced significantly soil pH, leading to a significant decrease on extractable As but to a significant increase on extractable metals. ‘Fe+Ca’ and ‘Fe+PM’ treatments were equally effective in reducing the extractable As fraction in the soils. Furthermore, as soil pH increased in those two treatments (‘Fe+Ca’ and ‘Fe+PM’ ) compared to ‘NA’ due to the liming effect provided by calcium carbonate and paper mill, extractable Cu and Zn were also significantly reduced.

The incorporation of FeSO₄ caused a toxic effect on plant growth, preventing their germination and growth of those seedlings which emerged (Table 3). ‘Ca’, ‘Fe+Ca’ and ‘PM’ treatments had a positive effect on plant growth in comparison to unamended soils due to the higher soil pH and the reduction of the soluble fraction of
metals. ‘Fe+PM’ was less effective in increasing plant biomass than ‘Ca’, ‘Fe+Ca’ and ‘PM’ treatments.

Dehydrogenase activity is considered an indicator of the average activity of soil microbial population, as these enzymes are an integral part of active microorganisms (Gil-Sotres et al. 2005). In this study, dehydrogenase activity has been positively affected by all the treatments, except ‘Fe’ (Figure 1). Also protease, the enzyme that catalyses protein hydrolysis to peptides and aminoacids (Ladd and Butler 1972; Alef and Nannipieri 1995c), was positively affected by the application of ‘Ca’, ‘Fe+Ca’ and ‘Fe+PM’. β-glucosidase activity was only significantly and positively affected by the application of ‘Fe’. Taking a general look over the effect of the amendments application on the enzymatic activity of the hydrolytic enzymes β-glucosidase, acid phosphatase, urease and cellulase, it is possible to conclude that the amendments induced a detrimental effect on their activities.

A principal component analysis (PCA) has been performed over the results in order to assess the relationships between enzymatic activities and physical, chemical and biological parameters. The output data will provide information about the soils properties which have been affected by the treatments and which have favoured or negatively affected the soils enzymatic activities.

Principal component analysis was carried out on 15 variables: soil pH, plant growth of A. elatius, plant growth of F. curvifolia (fresh biomass), extractable As, Cu, Mn, and Zn, available P and K, and the activities of dehydrogenase, β-glucosidase, cellulase, acid phosphatase, protease and urease. As there were no significant differences in the total trace element concentration and soil OM content among treatments at the end of the experiment, these variables were not included in the principal component analysis. The 15 variables were reduced to two principal
components. Principal component 1 (PC1) explained 60% of the variance, while principal component 2 (PC2) explained 15% of the variance, so together they explained about 75% of the total variance (Table 4). Both plants growth, soil pH, available P and K, extractable As and dehydrogenase activity had large positive loading coefficients on PC1, while β-glucosidase, extractable concentrations of Cu, Mn and Zn had negative factor loadings on PC1. Phosphatase, cellulase and urease were positively correlated with PC2 and protease activity was negatively correlated with PC2.

Figure 2 shows the combined plot of scores and loadings on PC1 versus PC2. ‘PM’, ‘Fe+Ca’ and ‘Ca’ were the treatments located on the most positive part of the PC1 axis, indicating that their application contributed to an increase in the properties with a high correlation with that axis (soil pH, extractable P and K, extractable As, plant growth and dehydrogenase activity), while ‘Fe’ was the treatment located on the most negative part of PC1, meaning that it had an opposite behaviour to the other treatments, contributing to a decrease in soil pH, extractable P and K, extractable As, plant growth and impaired dehydrogenase activity, and an increase in extractable Mn, Cu and Zn, and β-glucosidase activity. One interesting observation to be made is that dehydrogenase activity was less negatively affected by the increase in extractable As, than positively favoured by the increase of soil pH, as the three variables fitted well on the same component (PC1). Stated in a different way, the acidity correction of a soil seems to exert a stronger influence in the dehydrogenase activity than other factors.

Plants growth was positively affected by the addition of all the amendments which promoted an increase in soil pH. It is known that plant roots can create a favourable environment to microorganisms. Alvarenga et al. (2009) highlighted that the enzyme activity can be favoured by plant presence, although it cannot be assumed a concomitant plant yield improvement. In the present study, only one enzymatic activity
was positively correlated with plant growth. As expected, the increase in available P and K resulted to be beneficial to plant growth, which was corroborated by the fact that those properties were both positively correlated to PC1.

β-glucosidase is an extracellular enzyme, related to the C-cycle, which is involved in the organic matter mineralisation. The rise in metal extractability (Mn, Cu and Zn) did not act in detriment of β-glucosidase activity, as those soil characteristics fitted well together, with high negative factor loadings on PC1. This result is in accordance with those reported by other authors, who showed that soluble Cd and Zn did not alter the activity of β-glucosidase (Pérez de Mora et al. 2006). The increase of β-glucosidase activity with the incorporation of iron (II) sulphate is in agreement with other authors. Mench et al. (2006) observed a recovery of β-glucosidase activity after 7 years since iron-grit was applied, and Koo et al. (2012) reported that β-glucosidase activity increased over the control with the addition of FeSO₄ and iron grit separately. However, other authors stated that enzyme activities, such as β-glucosidase and cellulase, were inhibited by metals (Geiger et al. 1998).

Urease, cellulase and acid phosphatase activities were negatively affected by the application of ‘Ca’ and ‘Fe’, because both treatments appear in the most negative part of PC2. On the opposite, the ‘PM’ and ‘Fe+PM’ treatments were located in the most positive part of PC2, very close to the ‘NA’ treatments. As urease, cellulase and acid phosphatase activities were correlated with PC2, but not with PC1, no clear effects of soil pH, metals and As extractability on their activity has been possible to deduce. Consequently, it provides less information about the effects of contaminants on the ‘soil health’. Further, urease activity was less correlated to PC2, which can be attributed to the fact that this enzyme activity was less affected by the amendments applications.
From the results, considering that dehydrogenase activity showed the best positive correlation with plant growth, it could be selected as a good indicator of soil health improvement. Our conclusion is in agreement with other authors who stated dehydrogenase was a better indicator of the soil remediation progress than other enzymes (Pérez de Mora et al. 2005, 2006).

3.2 Effect of amendments on earthworm mortality and avoidance behaviour

The acute toxicity test with *E. fetida* was only able to detect some toxicity when the organisms were exposed to the soils with the ‘Fe’ treatment, with some mortality in the 100% exposition, but the results did not allow the calculation of the EC$_{50}$ values. The resilience of this organism to adverse conditions is well known, compromising the use of the acute toxicity test with *E. fetida* to detect low toxicity values (Alvarenga et al. 2008b, 2012). Taking these results into account, the avoidance behaviour tests in ‘Fe’ amended soils were carried out only with the 12.5; 25; and 50% (w/w) soil test dilutions with artificial soil, while the rest of the treatments, which did not induce earthworm mortality after 14 days of exposure, were tested using the complete set of dilutions, 12.5; 25; 50 and 100% (w/w) (Table 5).

In contrast to the mortality bioassay, the avoidance tests were more sensitive, showing that some of the treatments had their ‘habitat function’ impaired, with avoidance percentages higher or equal to 60. ‘Fe+PM’ treatment triggered a detrimental effect on the earthworms, which avoided the soil with that treatment in the dilutions tested. A limited habitat function, but to a lower degree, was also found in the ‘Ca’ treated soils for the 25% dilution, and in the 100% ‘NA’ and ‘Fe+Ca’ treatments. ‘PM’ was the treatment which improved the habitat function further with respect to the others.
The treatments which induced a higher toxic response in the *E. fetida* behaviour bioassays, ‘Ca’ and ‘Fe+PM’, triggered very different soil characteristics among those we have monitored: soil pH, extractable As and extractable metals, hampering a straightforward conclusion from this bioassay. Perhaps additional factors should be considered to elucidate this response, as the presence of other contaminants, the quality of the organic and inorganic fractions of the soil and the duration of the exposure. Natal da Luz et al. (2004, 2008) stated that a period of time longer than two days should be recommended to allow the earthworm’s response consolidation. Knoke et al. (1999) proposed that the way in which samples are prepared could affect the sensitteness of the test. Avoidance tests are considered valid assays to screen potential ecological risks, although an improvement of the method would help to reduce the variability of the response, and to understand how the results are influenced by other factors which may distort them (such as soil type, soil properties, etc.). Thus, properties of soil should be considered, as test and artificial soils do not share the same physical properties and can mimic the results (Alvarenga et al. 2012). It has been suggested the use of natural soil instead of artificial soil, without contaminant elements and with the same physical properties as the test soil (Jänsch et al. 2005). Thus, a recommendation to be taken in subsequent assays include the exclusion of the ‘soil properties’ from the influential factors set, using not contaminated natural soil with the same physical properties as the test soil instead of the artificial material.

3.3 Effect of amendments on *D. magna* and *V. fischeri*

EC$_{20}$ and EC$_{50}$ values, % (v/v), obtained in the acute tests with *D. magna* and *V. fischeri* are shown in Table 6, and the composition of the soil leachate used in these
tests is in Table 7. The only treatment which produced a soil leachate that was toxic towards *D. magna*, allowing the calculation of an EC$_{50}$ value, was ‘Fe’ treatment, as no immobilisation was observed in the other treatments. Bioassays carried out with *V. fischeri* did not allow the calculation of EC$_{50}$, only EC$_{20}$ values could be calculated.

‘Fe’ treatment was very toxic towards *D. magna*, recording a low EC$_{50}$ (9.8% v/v). In comparison with Alvarenga et al. (2007), Zn and Cu concentrations in the leachate from ‘Fe’ amended soil, which could elicit this toxic effect, were similar and higher, respectively.

Unamended soils, alongside with ‘Fe’ and ‘Fe+PM’ treatments, were found to be toxic to *V. fischeri*, being the ‘Fe’ treatment the most toxic as shown by the lowest EC$_{20}$ found. Furthermore, bioluminescence in the ‘Fe+PM’ treatment showed an increment of the EC$_{20}$ value after the second fraction of 15 minutes, which meant a recovery of the bacteria and a potential reversible effect of the toxicity after prolonged time of exposure. This recovery was observed neither in ‘Fe’ nor in ‘NA’ leachate. We can conclude that ‘Fe’ incorporation was the most toxic amendment for both organisms. All the other amendments had a beneficial effect towards *V. fischeri*, in comparison with the ‘NA’ treatment. The detrimental effects found on the behaviour of the organisms can be attributed to the metal(loids) concentration in the matrix (Alvarenga et al. 2007). Toxicity found towards *V. fischeri* could be related to the high Cu, Mn and Zn concentration leached found in both treatments with iron compared to the rest of treatments, including the unamended pots (‘Fe’ and ‘Fe+PM’, Table 7). EC$_{20}$ values in ‘Fe’ treatment towards *V. fischeri* were the lowest at 0.07 and 0.2 mg·L$^{-1}$ for Cu and Zn in the soil leachates, respectively. Alvarenga et al. (2007) found toxicity with similar Zn and lower Cu concentrations than those found in ‘Fe’ (0.13-0.2 mg·L$^{-1}$ for Zn and 0.0021-0.0015 mg·L$^{-1}$ for Cu). In the other toxic treatments (‘NA’ and ‘Fe+PM’),
metals concentrations required to cause a 20% diminution of the luminescence were lower in comparison with ‘Fe’ (0.002 and 0.01 mg L\(^{-1}\) for Cu and Zn in NA; 0.007 and 0.03 mg L\(^{-1}\) for Cu and Zn in ‘Fe+PM’). Higher As concentrations in the leachates of the ‘NA’ treatment could partially explain the toxicity found in this treatment. Other authors have also reported higher values of metals which triggered toxicity (Renoux et al. 2001; Tsiridis et al. 2006). It is important to notice that the results found in the studies mentioned above reported values of EC\(_{50}\), which means the leachates derived from the soils were more toxic than the ones in the present study, which only allowed the calculation of EC\(_{20}\) values. Moreover, one could assume that the low pH in the treatments which recorded EC\(_{20}\) values could be the primary cause of the reduction of V. fischeri luminescence. To test that, bioassays were also performed with pH correction (7.0±0.1) as it is specified in the method (ISO 11348-2 1998). As the toxic effect was also reported, it is possible to conclude that the toxicity was not due only to the acidity of the leachate.

As highlighted by other authors, no single bioassay is sufficient to monitor a remediation process, since organisms’ response towards toxicity is complex and diverse. It is essential to provide a battery of bioassays and select the most reliable ones to assess the remediation process and the toxicity of the contamination (Marwood et al. 1998). Chaîneau et al. (2003) and Alvarenga et al. (2008a) found that bioassays with V. fischeri and plant growth were very sensitive indicators, being more reliable to evaluate the reduction of adverse effects caused by toxicants than other bioassays, such as earthworm survival or seed germination. In the current study, although plant biomass was higher in ‘Fe+PM’ than in the ‘NA’ treatments, bioassays showed that this amendment addition does not create suitable conditions to the organisms assayed. At the
same time, treatments which reduced substantially metal extractable concentrations were found to trigger toxic effect on the earthworms.

4 Conclusions

Toxicity towards the organisms used in the bioassays was partly associated to the metal(loid) concentration in the soil, treated and non-treated, and in the leachates and, also, to the soil pH. It is recommended to use a battery of bioassays to evaluate the remediation success. Avoidance behaviour of *E. fetida* and *V. fischeri* luminescence were the most sensitive indicators of soil toxicity, so they should be used in future remediation processes to assess the effects of the amendment application in our area of study. Dehydrogenase should be measured, together with those two bioassays, as it provided reliable information when ameliorants, which promoted an improvement in soil quality, were added to the contaminated soils.

According to the results, the soil ‘habitat function’ in the unamended soil is impaired. The requirement for an intervention in the area of study and the incorporation of amendments within the soil in order to improve its quality is essential. However, not all the treatments are equally acceptable. The most promising amendments, able to create favourable conditions to the organisms used in this study to assess the soil quality improvement, are: the combined application of FeSO$_4$ 1% w/w and CaCO$_3$ 1% w/w, although further doses should be tested again to eliminate undesirable effects, such as the ones observed in the avoidance tests with *E. fetida*.

The treatment with FeSO$_4$ 1% w/w was the ‘worst case scenario’ tested: although it was able to reduce extractable As, it increased the extractable Cu, Mn and Zn concentration, which was a consequence of the decrease in soil pH that the application
of that amendment caused. As a consequence, this treatment had a detrimental effect in
some of the soil enzymatic activities (e.g. dehydrogenase, acid phosphatase, urease and
cellulase), did not allow plant growth, induced *E. fetida* mortality in the highest
concentration tested (100% w/w), and its soil leachate was very toxic towards *D. magna*
and *V. fischeri*. The combined application of FeSO$_4$ 1% w/w with other treatments (e.g.
CaCO$_3$ 1% w/w and paper mill 1% w/w) allowed a decrease in extractable As and
metals, and a soil pH value closer to neutrality. As a consequence, dehydrogenase
activity, plant growth and some of the bioassays identified those as better soil
treatments to this type of multi-contaminated soil.

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Figure captions

Fig. 1
Soil enzymatic activities (dehydrogenase, β-glucosidase, cellulase, acid phosphatase, protease and urease, mean±SE, n=8). Different letters show significant differences among treatments (p < 0.05). ‘NA’: not amended; ‘Fe’: FeSO₄; ‘Ca’: CaCO₃; ‘Fe+Ca’: FeSO₄+CaCO₃; ‘PM’: paper mill; ‘Fe+PM’: FeSO₄+paper mill.

Fig. 2
Scores of each sample on the two main principal components. PC1, first principal component; PC2, second principal component; ‘NA’: not amended; ‘Fe’: FeSO₄; ‘Ca’: CaCO₃; ‘Fe+Ca’: FeSO₄+CaCO₃; ‘PM’: paper mill; ‘Fe+PM’: FeSO₄+paper mill. As, Cu, Mn and Zn concentrations extractable with ammonium sulphate 0.1 M; available P and K. Each treatment was replicated four times, generating four points on the PCA plot.
Fig. 1.
Fig. 2.

Table 1 Uncontaminated soil, contaminated material, mixture 60:40 (w/w) (60% uncontaminated soil + 40% contaminated material) and paper mill chemical characterisation (mean±SE; n=3). n.d.: not-detected; n.a.: not analysed.

<table>
<thead>
<tr>
<th></th>
<th>Uncontaminated soil</th>
<th>Contaminated material</th>
<th>Mixture 60:40</th>
<th>Paper mill</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>6.02±0.01</td>
<td>4.1±0.01</td>
<td>4.9±0.03</td>
<td>7.7 ± 0.01</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>3.9±0.06</td>
<td>0.6±0.01</td>
<td>3.3±0.04</td>
<td>29.3 ± 0.40</td>
</tr>
<tr>
<td>Electrical conductivity (µS·cm⁻¹)</td>
<td>14.2±1.2</td>
<td>46.3±6.2</td>
<td>37.0±0.1</td>
<td>253.0±9.6</td>
</tr>
</tbody>
</table>

**Element pseudo-total concentration (mg·kg⁻¹)**

<table>
<thead>
<tr>
<th>Element</th>
<th>Uncontaminated soil</th>
<th>Contaminated material</th>
<th>Mixture 60:40</th>
<th>Paper mill</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>17.3±4.8</td>
<td>4265.8±506.1</td>
<td>1595.9±33.9</td>
<td>0.31±0.07</td>
</tr>
<tr>
<td>Cu</td>
<td>27.9±1.9</td>
<td>758.1±178.1</td>
<td>327.1±35.3</td>
<td>151.8±6.3</td>
</tr>
<tr>
<td>Mn</td>
<td>267.1±62.8</td>
<td>219.9±19.6</td>
<td>207.5±19.9</td>
<td>85.4±18.0</td>
</tr>
<tr>
<td>Zn</td>
<td>42.5±8.3</td>
<td>2455.1±430.2</td>
<td>910.9±133.6</td>
<td>41.1±5.9</td>
</tr>
<tr>
<td>Cd</td>
<td>n.d</td>
<td>40.3±3.7</td>
<td>19.3±0.3</td>
<td>8.67±0.6</td>
</tr>
</tbody>
</table>

**Element extractable concentration (mg·kg⁻¹)**

<table>
<thead>
<tr>
<th>Element</th>
<th>Uncontaminated soil</th>
<th>Contaminated material</th>
<th>Mixture 60:40</th>
<th>Paper mill</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>n.d</td>
<td>7.4±0.3</td>
<td>2.1±0.04</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cu</td>
<td>n.d.</td>
<td>10.0±0.1</td>
<td>1.9±0.05</td>
<td>n.a.</td>
</tr>
<tr>
<td>Mn</td>
<td>2.7±0.06</td>
<td>4.5±0.5</td>
<td>3.9±0.1</td>
<td>n.a.</td>
</tr>
<tr>
<td>Zn</td>
<td>0.7±0.1</td>
<td>21.1±0.3</td>
<td>8.7±0.05</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cd</td>
<td>n.d</td>
<td>1.3±0.5</td>
<td>0.06±0.01</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
Table 2  Soil organic matter (OM, %) soil pH, pseudo-total As and metal concentrations (Tot) in µg·g⁻¹, extractable As and metals concentrations (Ext) with (NH₄)₂SO₄ 0.1 M in µg·g⁻¹; available P (mg P₂O₅·Kg⁻¹), available K (mg K₂O·Kg⁻¹) at the end of the experiment (mean±SE, n=4). Values in a column marked with the same letter were not significantly different among treatments (p > 0.05). There were not significant differences among treatments for OM and pseudo-total concentration of trace elements (p > 0.05 for OM and p > 0.001 for metal(loid)s). n.d.: not-detectable. ‘NA’: not amended; ‘Fe’: FeSO₄; ‘Ca’: CaCO₃; ‘Fe+Ca’: FeSO₄+CaCO₃; ‘PM’: paper mill; ‘Fe+PM’: FeSO₄+paper mill.

<table>
<thead>
<tr>
<th>Treat.</th>
<th>OM</th>
<th>Soil pH</th>
<th>Tot-As</th>
<th>Tot-Cu</th>
<th>Tot-Mn</th>
<th>Tot-Zn</th>
<th>Tot-Cd</th>
<th>Ext-As</th>
<th>Ext-Cu</th>
<th>Ext-Mn</th>
<th>Ext-Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>2.6±0.1</td>
<td>5.0±0.03</td>
<td>1417.1±63.0</td>
<td>2984±45.2</td>
<td>254.6±12.9</td>
<td>859.3±117.4</td>
<td>5.9±0.6</td>
<td>2.7±0.1 a</td>
<td>2.5±0.06 b</td>
<td>6.2±0.4 c</td>
<td>8.6±0.7 a</td>
</tr>
<tr>
<td>Fe</td>
<td>2.9±0.1</td>
<td>3.4±0.02</td>
<td>1901.1±831.0</td>
<td>413.6±33.4</td>
<td>380.1±92.1</td>
<td>1128.1±429.0</td>
<td>18.7±8.3</td>
<td>0.4±0.03 c</td>
<td>8.4±0.9 a</td>
<td>65.7±22.5 a</td>
<td>16.6±3.0 a</td>
</tr>
<tr>
<td>Ca</td>
<td>2.6±0.2</td>
<td>8.2±0.07</td>
<td>1132.4±323.7</td>
<td>231.1±62.3</td>
<td>364.6±61.8</td>
<td>925.0±172.8</td>
<td>12.5±2.2</td>
<td>3.6±0.3 a</td>
<td>0.2±0.08 c</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Fe+Ca</td>
<td>2.7±0.1</td>
<td>7.4±0.04</td>
<td>2317.4±761.6</td>
<td>274.7±52.6</td>
<td>263.6±21.6</td>
<td>650.0±71.4</td>
<td>6.8±0.7</td>
<td>1.7±0.4 b</td>
<td>0.3±0.1 c</td>
<td>0.9±0.3 d</td>
<td>0.2±0.1 c</td>
</tr>
<tr>
<td>PM</td>
<td>2.4±0.03</td>
<td>7.4±0.08</td>
<td>2032.7±400.5</td>
<td>188.0±42.7</td>
<td>202.2±11.4</td>
<td>906.7±410.1</td>
<td>5.6±1.7</td>
<td>2.9±0.06 a</td>
<td>0.1±0.06 c</td>
<td>n.d</td>
<td>0.13±0.10 c</td>
</tr>
<tr>
<td>Fe+PM</td>
<td>2.9±0.2</td>
<td>6.6±0.10</td>
<td>1590.7±371.5</td>
<td>248.9±64.9</td>
<td>238.5±24.3</td>
<td>659.5±200.5</td>
<td>6.9±1.6</td>
<td>0.2±0.02 d</td>
<td>0.4±0.5 c</td>
<td>28.8±3.9 b</td>
<td>2.1±1.0 b</td>
</tr>
</tbody>
</table>
Table 3 Fresh weight biomass (g·pot⁻¹) of *A. elatius* and *F. curvifolia* in each treatment (mean±SE, n=4). Values in each of the two columns marked with the same letter were not significantly different among treatments (p > 0.05). ‘NA’: not amended; ‘Fe’: FeSO₄; ‘Ca’: CaCO₃; ‘Fe+Ca’: FeSO₄+CaCO₃; ‘PM’: paper mill; ‘Fe+PM’: FeSO₄+paper mill.

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>A. elatius</em></th>
<th><em>F. curvifolia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>1.5±0.1 b</td>
<td>0.4±0.05 c</td>
</tr>
<tr>
<td>Fe</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ca</td>
<td>2.5±0.04 a</td>
<td>0.8±0.05 a</td>
</tr>
<tr>
<td>Fe+Ca</td>
<td>2.3±0.06 a</td>
<td>0.8±0.07 a</td>
</tr>
<tr>
<td>PM</td>
<td>2.4±0.09 a</td>
<td>0.8±0.05 a</td>
</tr>
<tr>
<td>Fe+PM</td>
<td>1.5±0.07 b</td>
<td>0.6±0.05 b</td>
</tr>
</tbody>
</table>
Table 4 Factor loadings of each variable along PC1 and PC2, which resulted from principal component analysis (PCA). PC1, first principal component; PC2, second principal component. Correlations are significant when values were above 0.4.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. elatius</td>
<td>0.974</td>
<td>0.021</td>
</tr>
<tr>
<td>F. curvifolia</td>
<td>0.950</td>
<td>0.066</td>
</tr>
<tr>
<td>Soil pH</td>
<td>0.968</td>
<td>-0.131</td>
</tr>
<tr>
<td>Available K</td>
<td>0.963</td>
<td>0.148</td>
</tr>
<tr>
<td>Available P</td>
<td>0.917</td>
<td>-0.007</td>
</tr>
<tr>
<td>Extractable Mn</td>
<td>-0.810</td>
<td>-0.178</td>
</tr>
<tr>
<td>Extractable Cu</td>
<td>-0.935</td>
<td>-0.237</td>
</tr>
<tr>
<td>Extractable Zn</td>
<td>-0.931</td>
<td>-0.073</td>
</tr>
<tr>
<td>Extractable As</td>
<td>0.658</td>
<td>-0.058</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>0.709</td>
<td>-0.002</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>-0.810</td>
<td>0.404</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>0.139</td>
<td>0.742</td>
</tr>
<tr>
<td>Urease</td>
<td>-0.112</td>
<td>0.636</td>
</tr>
<tr>
<td>Protease</td>
<td>0.517</td>
<td>-0.546</td>
</tr>
<tr>
<td>Cellulase</td>
<td>0.422</td>
<td>0.808</td>
</tr>
<tr>
<td>Eigen Value</td>
<td>9.031</td>
<td>2.201</td>
</tr>
<tr>
<td>Explained variance (%)</td>
<td>60</td>
<td>15</td>
</tr>
</tbody>
</table>
Table 5 Avoidances percentages of *E. fetida* towards the test soil. * is significant at p < 0.05; n.av.: non avoidance behavior was found. ‘NA’: not amended; ‘Fe’: FeSO₄; ‘Ca’: CaCO₃; ‘Fe+Ca’: FeSO₄+CaCO₃; ‘PM’: paper mill; ‘Fe+PM’: FeSO₄+paper mill.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>n.av.</td>
<td>n.av.</td>
<td>n.av.</td>
<td>70*</td>
</tr>
<tr>
<td>Fe</td>
<td>n.av.</td>
<td>n.av.</td>
<td>20*</td>
<td>-</td>
</tr>
<tr>
<td>Ca</td>
<td>35*</td>
<td>65*</td>
<td>23*</td>
<td>n.av.</td>
</tr>
<tr>
<td>Fe+Ca</td>
<td>n.av.</td>
<td>n.av.</td>
<td>n.av.</td>
<td>69*</td>
</tr>
<tr>
<td>PM</td>
<td>n.av.</td>
<td>n.a</td>
<td>n.av.</td>
<td>n.av.</td>
</tr>
<tr>
<td>Fe+PM</td>
<td>60*</td>
<td>75*</td>
<td>60*</td>
<td>85*</td>
</tr>
</tbody>
</table>
Table 6: Acute toxicity tests towards *D. magna* and *V. fischeri*. EC₅₀ and EC₂₀ values calculated with *D. magna*'s immobilisation and *V. fischeri*'s luminescence. n.t.: no detected toxic effect. ‘NA’: not amended; ‘Fe’: FeSO₄; ‘Ca’: CaCO₃; ‘Fe+Ca’: FeSO₄+CaCO₃; ‘PM’: paper mill; ‘Fe+PM’: FeSO₄+paper mill.

<table>
<thead>
<tr>
<th>Water-leachates</th>
<th>Immobilisation <em>(D. magna)</em></th>
<th>Luminescence inhibition <em>(V. fischeri)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (% v/v)</td>
<td>EC₂₀ (% v/v)</td>
</tr>
<tr>
<td>NA</td>
<td>n.t.</td>
<td>21.24</td>
</tr>
<tr>
<td>Fe</td>
<td>9.8</td>
<td>13.37</td>
</tr>
<tr>
<td>Ca</td>
<td>n.t.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fe+Ca</td>
<td>n.t.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PM</td>
<td>n.t.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fe+PM</td>
<td>n.t.</td>
<td>11.88</td>
</tr>
</tbody>
</table>
Table 7 Soil leachate (ratio 1:10 soil to water, w/v) characteristics (mean, n=4): pH, As and metals concentrations (mg·L⁻¹). ‘NA’: not amended; ‘Fe’: FeSO₄; ‘Ca’: CaCO₃; ‘Fe+Ca’: FeSO₄+CaCO₃; ‘PM’: paper mill; ‘Fe+PM’: FeSO₄+paper mill.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>As</th>
<th>Cu</th>
<th>Mn</th>
<th>Zn</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>6.22</td>
<td>0.223</td>
<td>0.013</td>
<td>0.034</td>
<td>0.072</td>
<td>0.025</td>
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<tr>
<td>Fe</td>
<td>3.81</td>
<td>0.024</td>
<td>0.529</td>
<td>5.64</td>
<td>1.623</td>
<td>0.172</td>
</tr>
<tr>
<td>Ca</td>
<td>7.62</td>
<td>0.841</td>
<td>0.014</td>
<td>0.004</td>
<td>0.052</td>
<td>0.030</td>
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<tr>
<td>Fe+Ca</td>
<td>7.40</td>
<td>0.088</td>
<td>0.001</td>
<td>0.057</td>
<td>0.050</td>
<td>0.019</td>
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<tr>
<td>PM</td>
<td>7.11</td>
<td>0.922</td>
<td>0.020</td>
<td>0.011</td>
<td>0.046</td>
<td>0.065</td>
</tr>
<tr>
<td>Fe+PM</td>
<td>6.65</td>
<td>0.054</td>
<td>0.029</td>
<td>2.644</td>
<td>0.147</td>
<td>0.055</td>
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