Toxicity in lead salt spiked soils to plants, invertebrates and microbial processes: unraveling effects of acidification, salt stress and ageing reactions

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Abstract

The fate and effects of toxic trace metals in soil freshly spiked soluble metal salts does not mimic those of metals in the field. This study was set up to test the magnitude of effects of salinity, acidification, and ageing on toxicity of lead (Pb) to plants, invertebrates and soil microbial processes. Three soils were spiked with Pb^{2+} salts up to a concentration of 8000 mg Pb/kg and were tested either after spiking, after soil leaching followed by pH correction, or after a 5-year outdoor ageing period with free drainage followed by pH correction. Soil solution ionic strength exceeded 150 mmol/L in soils tested directly after spiking and this decreased partially after leaching and returned back to background values after 5 y outdoor equilibration. Chronic toxicity to two plants, two invertebrates, and three microbial endpoints was consistently found in all spiked soils that were not leached. This toxicity significantly decreased or became absent after 5 years of ageing in 19 of the 20 toxicity tests by a factor 8 (median factor; range: 1.4->50), measured by the factor increase of total soil Pb dose required to induce 10% inhibition. The toxicity of Pb in leached soils was intermediate between the other two treatments. The lowest detectable chronic thresholds (EC10) in aged soils ranged 350-5300 mg Pb/kg. Correlation analysis , including data of Pb^{2+} speciation in soil solution, suggest that reduced ionic strength rather than acidification or true ageing is the main factor explaining the soil treatment effects after spiking. It is suggested that future toxicity studies should test fine PbO powder as a relevant source for Pb in soils to exclude the confounding salt effects.

Highlights

Lead toxicity in freshly spiked, unleached soils is primarily confounded by salinity stress and toxic thresholds of Pb in aged and fully leached soils are found at relatively large concentrations with lowest effect thresholds found for earthworms.

Keywords

Lead; Soil toxicity; Bioavailability; Phosphorus; Spiking;

Introduction

Lead (Pb) is probably the first metal extracted from its ores by man and its widespread use since Roman times led to extensive environmental soil pollution (Steinnes, 2013). The human health effects are well documented but effects on soil-dwelling organisms due to soil Pb contamination have been surprisingly difficult to identify in the field. For example, in soils sampled at shooting ranges with total soil Pb concentrations up to 2400 mg Pb/kg, toxic effects to the springtail *Folsomia candida* or to the enchytraeid *Enchytraeus crypticus* were more related to the acid soil pH than to elevated soil Pb (Luo et al., 2014a; Luo et al., 2014b). Lead occurs as the Pb^{2+} ion that has the greatest binding strength in soil among the most commonly studied toxic metals Cd, Cu, Co, Zn, Ni, Pb (Degryse et al., 2009) and the strong immobilisation of Pb^{2+} is perhaps explaining the relatively low toxicity to organisms exposed to soil Pb via the soil solution.

There is a reasonably large set of Pb toxicity data from laboratory studies conducted using soils freshly spiked with Pb^{2+} salts. Such laboratory data suggest that Pb toxicity occurs near the natural soil background range of Pb (2-200 mg Pb/kg). No Observed Effect Concentrations (NOECs) or 10% effect concentrations (EC10) can be found as low as 50 or

100 mg/kg (added Pb) for barley and oat (Aery and Jagetiya, 1997; Khan and Frankland, 1984) 129 mg/kg for earthworms (Bentsson et al., 1986) and 150 to 200 mg/kg for soil microbial respiration and N-mineralization (Chang and Broadbent, 1982; Doelman and Haanstra, 1984). The toxicity in soils freshly spiked with soluble metal salts overestimate toxicity in corresponding field-contaminated soils due to lack of sufficient equilibration time in the spiked soils (lack of ageing) and to confounding factors such as higher salinity and acidification. For different metals, empirical 'leaching-ageing' or 'lab-to-field' factors translating that difference have been identified in toxicity tests and adopted in risk assessment, however, for Pb this factor is not well established (Smolders et al., 2009). The toxicity of Pb to *F. candida* in environmentally contaminated soils was compared with corresponding soils spiked with $Pb(NO_3)_2$ (Lock et al., 2006). The Pb doses required to reduce the reproduction of *F. candida* in freshly spiked soils by 50% ranged 2200-3200 mg Pb/kg and corresponding doses in the field contaminated soils were at least a factor of two larger. In a field trial conducted in Nagyhörcsök (Hungary) in 1991(Kádár et al., 1998), Pb was applied as $Pb(NO_3)_2$ at three rates with the highest application rate equivalent to about 250 mg added Pb/kg soil. During the first year after application, the grain yield of maize was significantly reduced by 28% at the highest Pb application whereas toxic effects disappeared in subsequent years.

The fraction of isotopic exchangeable metal in soil is a suitable index to identify the 'ageing' reaction and to denote the difference in metal (Zn, Cu) toxicity between soils freshly spiked with metal salts and well equilibrated soils or field-contaminated soils (Hamels et al., 2014). No such comparison between isotopic exchangeable metals and metal toxicity have yet been made for Pb but the chemical data suggest that the ageing reactions of Pb are not strongly

93 pronounced. For example, the isotopically exchangeable Pb fraction is only 2-fold larger in
94 freshly spiked soil compared to field contaminated soils (Degryse et al., 2007). An extensive
95 survey in a British catchment affected by Pb mining showed that the isotopically exchangeable
96 Pb fraction was 80% in most acid soils, decreasing to about 30% near pH 7 (Marzouk et al.,
97 2013). This study also showed that Pb was clearly more labile than zinc. Soils contaminated by
98 petrol-derived Pb also had somewhat larger isotopically exchangeable Pb fractions than soils
99 in which Pb was derived from sewage sludge application or from Pb-containing minerals (Mao
100 et al., 2014).
101
102 Toxicity in Pb^{2+}-salt spiked soils is confounded by the associated pH decrease (Speir et al.,
103 1999) which results from the displacement of protons by Pb^{2+} on the sorption surfaces. In
104 addition, application of Pb^{2+} salts (e.g., $PbCl_2$) increases the salinity of the soil solution and
105 may induce salinity stress (Stevens et al., 2003). These factors do not occur where atmospheric
106 deposition of the alkaline PbO (e.g., Pb smelters) is the source of soil Pb or where the
107 emissions are gradual, thereby allowing time for leaching of excess salts. The confounding
108 effects of salinity and acidification on metal toxicity are found for all metals but these
109 confounding factors become increasingly important for those metals where large doses, e.g.
110 >20 mmol divalent metal/kg soil, are required to elicit a response. Such might be the case for
111 Pb because of its large immobilization in soil. It was calculated that leaching is essential for
112 the identification of genuine Pb toxicity to plants in soils with pH>5 where strong Pb^{2+} sorption
113 requires high Pb^{2+}salt doses to invoke toxicity (Stevens et al., 2003) Leaching, however, does
114 not remove the acidification induced by the sorption of Pb^{2+} on the variable charge binding
115 sites in soil. Leaching of soil reduced toxicity of copper (Cu) salt amended soils to barley
116 seedlings and it was shown that the additional Ca uptake in non-leached soils (due to

117 increased solution Ca^{2+} in spiked, non-leached soils) contribute to the confounding factors
118 (Schwertfeger and Hendershot, 2013b).
119
120 This study was designed to compare Pb toxicity between well equilibrated, leached, and pH-
121 corrected soils and soils freshly spiked with Pb^{2+} salts and to identify factors involved in that
122 difference. Lead toxicity in spiked soils was tested in soils under three treatments after spiking,
123 i.e. spiked, spiked + leached + pH corrected, or aged (5 years) after spiking with leaching and
124 pH correction. These different treatments allow the separation of the different factors
125 altering toxicity (i.e., salinity, acidification, equilibration time) and may suggest which soil
126 manipulations are required for normalizing the results of Pb toxicity tests conducted in freshly-
127 spiked to field-contaminated soils. The toxicity tests in this study included a variety of
128 organisms (plants, soil microbial processes and invertebrates) to cover a range of organisms
129 with potentially different exposure routes.
130
131 **Materials and Methods**
132 *Experimental design*
133 Three different soils were spiked with Pb salts and toxicity was compared among three
134 treatments with stepwise increasing complexity, i.e. (A) freshly spiked soils, (B) spiked soils
135 which are leached and pH corrected and (C) spiked but aged soils, the latter including the
136 leaching and pH correction. Toxicity was measured with six different assays (7 endpoints) abd
137 thresholds are reported as metal concentrations measured in soils after each soil treatment.
138
139 *Soil sampling and characterization*
140

141 Three uncontaminated topsoils with varying soil properties were collected from Spain (BA),
142 the United Kingdom (WB) and Belgium (TM). The soil BA was from arable land and classified
143 as calcic luvisol, contained 16% clay and 10% $CaCO_3$; soil WB was from grassland and classified
144 as dystric luvisol, containing 30% clay. Soil TM was from arable land and classified as haplic
145 luvisol with 12% clay. Soils were collected with a metal spade from the plough layer, or for
146 grassland, from the surface horizon after clearance of the grass thatch layer. The time
147 between sampling and cold storage was never more than one week, followed by storage at
148 4°C until drying. The soils were air-dried, sieved through a 4-mm sieve, and stored at room
149 temperature prior to soil characterization and spiking.
150
151 The carbon concentration in soil was measured by ignition with an elemental analyzer (EA112,
152 CE instruments). Organic carbon was calculated as the difference between total C and
153 carbonate C. The carbonate C was determined from pressure increases after addition of HCl
154 to the soil in closed containers (including $FeSO_4$ as a reducing agent). Soil moisture at pF 0
155 (saturation) and pF 1.9 (80 cm suction) was determined by the sandbox method using 100 cm^3
156 soil cores (P1.80-1, Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands). Aqua
157 regia–soluble metal concentrations in all soil treatments, including unspiked soils, were
158 determined by boiling aqua regia extractions of 0.1 g homogenized samples and analysis of
159 the digest solutions by inductively coupled plasma/optical emission spectroscopy (ICP-OES,
160 Perkin-Elmer Optima 3300 DV, Norwalk, CT, USA). Certified reference materials BCR-142R
161 (uncontaminated light sandy soil, Institute for Reference Material Measurement, Joint
162 Research Center, European Commission, certified value 25.7 ± 1.6 mg Pb/kg; measured value
163 24.0 ± 1.9 mg Pb/kg; recovery ≈93%) and BCR-143R (sewage sludge contaminated soil,
164 Institute for Reference Material Measurement, Joint Research Center, European Commission,

165 certified value 174 ± 5 mg Pb/kg; measured value 165 ± 8 mg Pb/kg; recovery ≈95%) were
166 included. The soil pH was measured in 0.01 M $CaCl_2$ (1:5 soil/solution ratio) after shaking for
167 2 h and allowing to settle for 10 min before pH measurement. The silver-thiourea method
168 (Chhabra et al., 1975)was used to measure the effective cation exchange capacity (eCEC; at
169 soil pH) and exchangeable cations, with concentrations in extracts determined by ICP-OES. Soil
170 properties are given in Table 1.
171
172 *Soil spiking, leaching, and pH correction*
173
174 Air-dried and sieved uncontaminated soil samples were spiked with $Pb(NO_3)_2$ (treatment C) or
175 $PbCl_2$ (other treatments; Table 1) to seven concentrations (control plus six treatments: 250,
176 500, 1000, 2000, 4000, and 8000 mg Pb/kg). Additional deionized water was added together
177 with the spike solution to adjust the soil moisture content to 75% of pF 2.0. The $PbCl_2$ solubility
178 limit required Pb dosing as crushed powder at the highest concentrations. We previously
179 demonstrated that the solid $PbCl_2$ salt dissolved in soil (Cheyns et al., 2012). All soils were
180 thoroughly mixed after amendments using laboratory spoons. Moisture content in control
181 soils was raised to 75% of pF 2.0 with deionized water only. One portion of the spiked soils
182 was air-dried, sieved again and stored pending toxicity testing. The other portion of the spiked
183 soils were incubated at 20°C for a week after which these were leached and pH corrected. The
184 leaching was performed as described by (Oorts et al., 2006) and is set-up to remove about one
185 pore volume. We used artificial rain water (ARW, $5*10^{-4}$ M $CaCl_2$, $5*10^{-4}$ M $Ca(NO_3)_2$, $5*10^{-4}$ M
186 $MgCl_2$, 10^{-4} M Na_2SO_4, 10^{-4} M KCl, pH 5.9) for leaching the soils. Soils were transferred to a
187 flowerpot (2.5 kg/pot) in which the perforated bottom was covered by a filter cloth (mesh size
188 140-150 µm). The flowerpot was then put in a 5L bucket filled with 1 L ARW allowing to

189 moisten the soil from bottom onwards. ARW was added to the bucket until the water surfaced
190 reached the soil surface in the flowerpot. Another pore volume ARW was then poured directly
191 in the flowerpot. Subsequently, the flowerpots were removed from the buckets, left to drain
192 for 24h, and the leached soils were homogenised, sieved and air-dried again for about 1 week.
193 After drying, pH was analysed in the leached soils. Soil pH values for each treatment were
194 adjusted with CaO to maintain soil pH within 0.2 pH units within each Pb concentration
195 gradient. The CaO was added as crushed powder and soils were incubated again for 1 week at
196 75% of pF 2. After sieving and drying, pH was measured again and soils were finally air dried
197 pending toxicity testing. Treatment C was based on soils that had been spiked with $Pb(NO_3)_2$
198 5 years before the other treatments commenced. Five kg (dry weight) of each Pb
199 concentration was stored in perforated flower pots, which were incubated outside (Leuven,
200 Belgium) in a sand box with free drainage. No pH correction was made before incubation
201 outside, but pH was corrected after ageing with CaO as described above. The excess water
202 drainage in Belgium is about 300 mm/year, theoretically yielding >10 pore volumes leaching
203 in 5 years in these flower pots.
204
205 *Soil solution analysis and Pb speciation*
206 At the time of plant growth testing, a subset of soils of each dose was incubated for four weeks
207 after rewetting and was analyzed for pore water composition. Soil solution was sampled by
208 the double chamber centrifugation method (30 min at 3000 g), through a 0.45 µm membrane
209 filter and pH was recorded. The elemental composition was measured by ICP-OES. Anion
210 concentrations were analyzed by anion chromatography (DIONEX, ICS-2000). Concentrations
211 of dissolved organic and inorganic carbon were measured by a TOC-analyzer (Multi N/C 2001
212 S, Analytic Jena). Soil solution ionic strength was calculated from Ca, Mg, K, Na, Cl^-, SO_4^{2-} , and

213 inorganic C concentrations. Lead free ion activities were modeled from soil solution
214 composition with an assemblage model (WHAM6, version 6.0.13, Natural Environment
215 Research Council). The measured pore water concentrations of Mg, Ca, Pb, Cl and dissolved
216 inorganic carbon (M) were entered into the model as total dissolved species. The pH of the
217 soil was used as input and temperature was set as 20 °C. The Fe^{3+} activity (M) was calculated
218 by the ion activity product of Fe hydroxide and the pH of the soil (log(Fe)=-3*pH+2.5). It was
219 assumed that 65% of dissolved organic matter is present as fulvic acids while the other 35 %
220 of DOM was inert material (Tipping et al., 2003).
221
222 *Toxicity tests*
223
224 All soil treatments (i.e., A: freshly spiked, B: leached+pH corrected, C: aged+leached+pH
225 corrected) were tested at the same time for an individual soil to minimize variation, however,
226 different soils and different assays were tested serially.
227
228 *Plant growth tests.* Tomato and barley were used as test plants and the endpoint was shoot
229 yield at 2 weeks after emergence (ISO 11269-2, 2012). The air-dried soils were fertilized with
230 50 mg P/kg soil as KH_2PO_4 and 100 mg N/kg as KNO_3. These fertilized soils were pre-incubated
231 at a moisture content equivalent to 75 % of that at pF 2.0. After a 14-d equilibration period,
232 non-porous plastic pots with a top internal diameter of 85 mm were filled with 450 g (dry
233 weight) of control soil and soils spiked with Pb establishing four replicates per treatment.
234 Summer barley (*Hordeum vulgare*; monocotyledonous) and tomato (*Lycopersicon esculentum*
235 Miller; dicotyledonous) were selected for the test. Twenty uniform, undressed seeds of the
236 selected species were planted in each pot and placed in a growth cabinet (Weiss, 18' SP/+5

237 Ju-Pa; 16 h/8 h light/darkness cycle, 20 °C during light hours and 16 °C during night time, 70%
238 humidity). As soon as 50 % of the control seedlings emerged, i.e. after 2-5 days of growth,
239 seedlings were thinned to give a total of five evenly spaced representative specimens of the
240 plants in the pots. After an additional 14 days of growth (i.e. 14 days after the day of >50%
241 emergence), shoot biomass above the soil surface was removed, and the dry mass of the
242 shoots/pot was determined after oven drying at 70°C for 16 h. Dry plant material was crushed,
243 digested and analyzed for elemental composition (ICP-OES).
244
245 *Microbial tests*. The effects of Pb on nitrification were assessed with a standard test in which
246 ammonium is added to the soil before incubation (ISO 14238, 1997). The percentage nitrate,
247 relative to the added ammonium, is measured after 28 days incubation. The endpoint is called
248 the Substrate Induced Nitrification or SIN. An extra measurement was included in this ISO test
249 since after 28 days there may be substrate (ammonium) limitation. The Potential Nitrification
250 Rate (PNR), which is the nitrification rate at unlimited substrate (NH_4^+) availability, was
251 measured after 7 days incubation. This adjustment in incubation time obtains a larger
252 sensitivity to added metals (Smolders et al. 2001). Prior to the nitrification test, the air-dried
253 soils were pre-incubated at a moisture content equivalent to 75% of that at pF 2.0 for 14 days
254 at 20°C. At the start of the experiment, soils were amended with 100 mg NH_4-N/kg fresh soil
255 using a stock solution containing 80 mg $(NH_4)_2SO_4$/mL (Smolders et al. 2001). The PNR (mg
256 NO_3-N/kg fresh soil/day) was calculated from the linear increase in soil NO_3-N between 0 and
257 7 days after substrate addition. The SIN was calculated after 28 days incubation as the
258 percentage added ammonia that was nitrified. For each soil, the SIN was determined using
259 the replicate NO_3-N concentrations at day 28 and the average (n = 3) NO_3-N concentrations at
260 day 0.

261

262 The second assay the Substrate Induced Respiration test (SIR). The OECD-217 carbon
263 transformation test (OECD 217, 2000) is such a test in we selected 24h respiration as an
264 endpoint. Prior to the respiration test, the air-dried soils were pre-incubated at a moisture
265 content equivalent to 75 % of that at pF 2.0 for 14 days at 20°C. After this incubation period 5
266 g subsamples at each Pb concentration (of each soil) were placed in 20 ml plastic pots. Soils
267 were then amended with 0.125 mL of ^{14}C labelled glucose solution (40 mg/mL glucose, specific
268 activity = 2.3 kBq/mg glucose-C) and mixed thoroughly. The plastic pots with soil were placed
269 in airtight Schott bottles of 100 ml with 5 ml of NaOH 1 N at the bottom. These Schott bottles
270 were incubated at 20°C in dark for 24 hours. Upon completion of the incubation period NaOH
271 traps were removed and 1.0 mL subsamples were taken and added to 10 mL scintillation
272 cocktail (Ultima Gold), with the solutions then shaken and activity determined by beta
273 counting. The percentage added glucose-C which was respired, was determined.

274

275 *Invertebrate tests.* The toxic effects of Pb to soil invertebrates were assessed using Collembola
276 (*Folsomia candida*) and earthworm (*Eisenia fetida*) reproduction tests (ISO 11267, 2014; OECD
277 222, 2004). Chronic toxicity tests with F. candida were conducted according to the guideline
278 where 10 synchronised collembola of 10 to 12 days old were exposed per glass vessel
279 containing 25 g (dry weight) of moist soil. The reproduction assay with *F. candida* lasts 28 days.
280 Granulated dry yeast (2 mg) was added on the soil surface as food on day 0 and day 14. At the
281 end of the test, juveniles were counted after extraction by flotation in water with a few drops
282 of blue ink. Digital images were taken after flotation and offspring and adults counted from a
283 hard copy of the image. During exposure, all test vessels were kept at 20±1°C at a 16h/8h
284 light/dark cycle at 400-800 lux. Soil moisture content was checked twice a week by weight loss

285 and replenished with the appropriate amount of deionised water, as necessary. In all tests, 10
286 replicates were tested per concentration. The second assays is the 28 days reproduction test
287 of the earthworm *Eisenia fetida* according to OECD 222 (2004). In the reproduction tests, 10
288 adult earthworms from a synchronized culture were exposed per glass test container
289 containing an amount of moist soil corresponding with 500 g dry weight. Mass of adult
290 earthworms was determined at the start of exposure (per 10 worms and for approx. 30-40
291 worms also individually). The earthworms received 5 g dry weight equivalents of moistened
292 horse dung for food (in a small hole made in the middle of the soil). After 4 weeks of exposure,
293 soils were hand sorted to remove the adult earthworms and assess survival. Soils were
294 returned into the test containers and incubated for an additional 4 weeks to allow cocoons to
295 hatch. Juveniles were counted by hand sorting from soil. During exposure, all test containers
296 were maintained at 20±1 °C at constant illumination with 400-800 lux. Soil moisture content
297 was checked twice per week by weight loss and replenished with the appropriate amount of
298 deionised water, when necessary. Additional food was added to test chambers, as necessary.
299 In all tests, 4 replicates were tested per concentration.
300
301 *Statistical analysis*
302
303 The responses of all assays were converted to relative response (RR, in %), that is the response
304 relative to that of the mean in the corresponding control soil. This conversion was made per
305 soil, test, and soil treatment after spiking. The dose-response relationships were fitted to the
306 log-logistic model (Doelman and Haanstra, 1989) using the Newton optimization for non-
307 linear regression (JMP Pro 11.2, SAS 2013)). The 'dose' in this model is the added Pb
308 concentration (measured concentration minus the background concentration), with the dose

in the control soil attributed a very small value (0.1 mg Pb/kg). To estimate the total soil Pb concentrations at 50% inhibition (EC50, mg Pb/kg soil) the model reads:

$$RR = \frac{100}{\{1+exp[b(\ln(dose)-\ln(ED50))]\}} \quad (1)$$

with b the slope and ED50 the dose (i.e., added Pb) at 50 % inhibition. The EC50 is

$$EC50 = ED50 + background \quad (2)$$

with background the total concentration of Pb in the control soil. Similarly, EC10 is fitted directly as:

$$RR = \frac{100}{\left\{1+\frac{1}{9}exp[b(ln(dose)-ln(ED10))]\right\}} \quad (3)$$

in which ED10 is the dose at 10% inhibition and EC10=ED10+background.

The starting parameter values for fitting Eqns. (1) and (3) were based on graphical evaluation with b values limited to 0.2-5.0.

The effects of soil treatment after spiking on toxicity were expressed as the treatment effects on corresponding ED10 values. This was quantified with the factors 'f' (dimensionless) defined as the ratio of ED10 from two treatments. Three different f values can be defined based on comparisons of treatments B with A, C with A or C with B (Fig.1). For example, the ratio of the ED10 of a given test in leached and aged soil to that in a corresponding freshly spiked soil yields the leaching-ageing factor for that test and soil, defined as f_3 (Fig.1). The majority of responses to soil Pb after the ageing were statistically insignificant or ED10 values had larger errors relative to that in freshly spiked soil, hence it was either not possible to calculate parameter f or to test if f was significantly different from 1.0. To address the issue, a stepwise procedure was followed. First, if the response curve after leaching or ageing was not

significant, it was assumed that the ED10 in that soil was higher than the highest added soil Pb concentration and

$$f > \frac{(highest\ tested\ concentration - background)_{leached\ or\ aged}}{ED10_{spiked}} \quad (4)$$

with the highest tested concentration the total soil Pb concentration in the leached or leached+aged soil. The value of f was defined significantly different from 1 if the upper range of 95% confidence interval of the ED10 was lower than the highest tested concentration corrected for background. Alternatively, if there was a significant treatment effect after leaching or ageing, f was estimated as a parameter in a non-linear model fitted to the *combined data* of, for example, the leached+aged soils and freshly spiked soils of a test in a soil:

$$RR = \frac{100}{\left\{1+\frac{1}{9}exp\left[b\left(ln(dose) - ln\left(ED10_{spiked}\right) - \ln(D \times f)\right)\right]\right\}} \quad (5)$$

with D a dummy variable =1 for leached or aged soils and 0 for freshly spiked soils. The factor f and its 95% confidence limits allow testing if f is significantly different from 1.0, i.e. if there is a significant difference in ED10 due to soil treatment. Equation (5) assumes that the slopes of the dose-response curves are equal for each pair of treatments and, hence, that the treatments have equal effects on the factor change in ED10 as in ED50. This assumption was tested on a few selected cases and b values were, on average, somewhat smaller (i.e. dose-response curves less steep) in leached and/or aged soils than in spiked soil but the confidence intervals of b typically overlapped. The slope parameter b in Eqn. (5) was first fitted on the spiked soil (most robust data) and was a fixed parameter for the subsequent fitting of parameter f_1 or f_3 and $ED10_{spiked}$ in the combined data set. Along the same lines, the factor f_2 due to ageing after leaching was found by first fitting b on the leached soils and using that slope as a fixed parameter in the subsequent fit.

354

355 **Results**

356

357 The recovery of added Pb in freshly spiked soils, measured by aqua regia extraction, was 98
358 ±19% (mean ± standard deviation) while it was 86 ±14% in spiked+leached+pH corrected soils
359 and 93 ±34% in the spiked+aged+pH corrected soils. Lead spiking decreased soil pH in the
360 freshly spiked soils by up to 1.4 pH units. In contrast, soil pH in the soils at the higher Pb doses
361 did not differ more than 0.2 pH units from the control values after soil liming (Fig. 2). The pore
362 water pH (data not shown) decreased by up to two pH units at the highest Pb concentrations
363 in the freshly spiked soils. Despite pH corrections, the pore water pH values decreased with
364 increasing Pb in the leached treatments, in contrast with soil pH values of these treatments.
365 This might be related to increased soil solution ionic strength at higher Pb doses (see below).
366 The pore water pH was unaffected by soil Pb in all aged and pH corrected soils. The pore water
367 ionic strength (IS) exceeded 150 mmol/L in the freshly spiked soil at highest $PbCl_2$ doses (Fig.2)
368 . Leaching of the soils (about 1 pore volume) after spiking strongly reduced pore water IS in
369 soil WB but only halved the IS in soil BA and had been almost ineffective in soil TM. The natural
370 leaching (>10 pore volumes) during the 5-year outdoor weathering completely removed the
371 effect of spiking on IS (Fig. 2). The soil solution Pb concentrations increased with increasing Pb
372 dose and ranked spiked>leached>aged except in soil BA where, surprisingly, soil solution Pb
373 was generally highest in the aged soils. This might be related to colloidal Pb since the low ionic
374 strength soil solutions of the aged soil BA contained relatively high concentrations of Fe and
375 Al. Lead forms stable complexes with colloidal Fe and Al (Pedrot et al., 2008). The free Pb^{2+} ion
376 activity (data not shown) only exceeded 1 µM in the freshly spiked soil TM. After leaching, the

377 corresponding maximum was 0.3 µM in that soil and was below 0.1 µM in all other treatments
378 and soils at all spiked Pb levels, illustrating strong Pb^{2+} sorption capacity in all soils.
379
380 The absolute values of the biological responses in the uncontaminated control soils are given
381 in Table SI1. There were significant differences in these responses among soils and soil
382 treatments for some tests. For example, the leaching procedure reduced plant growth in the
383 control soil of soil BA but not in the other soils. The nitrification rate PNR was lower in soil TM
384 than in the other two soils. For that reason, all responses are expressed relative to the
385 corresponding control. Selected dose response curves are shown in Fig. 3. Toxicity was
386 significant and pronounced for all tests in all freshly spiked soils, with lowest EC50 at 480 mg
387 Pb/kg for earthworms in soil BA. In contrast, toxicity in the aged soils was much less
388 pronounced, yielding only five detectable EC50 values for 20 tests (Table 2) and detectable
389 EC10 values in 10 of the 20 tests (Table 3). The lowest EC50 in aged soils was 1270 mg Pb/kg
390 (earthworm, soil BA). The toxicity in leached and pH corrected soils was intermediate to
391 freshly spiked and aged soils. The factor change in toxicity due to leaching, pH correction, and
392 ageing relative to freshly spiked soils (f_3) was significantly above 1.0 in 19 of the 20 tests,
393 indicating an overall reduced toxicity compared to freshly spiked soils (Table 3). The median
394 factor f_3 among soils and tests was factor 8 when including unbounded values (i.e. the lower
395 estimate of that value). The f_3 ranged 1.4-65 based on the significant curves, potentially
396 reaching higher values based on the unbounded values. The corresponding median factors
397 due to leaching/pH correction only was 2 (f_1) and due to ageing was 3 (f_2). Leaching/pH
398 correction effects or ageing effects were not consistently significant (Table 3). The average
399 leaching/pH correction factor was lowest for soil TM for which the leaching had not been
400 successful in reducing ionic strength. Finally, the aggregate response curves for all tests and

401 soils illustrates significantly reduced toxicity which ranked spiked>leached>aged soils (Fig. 4).
402 Correlation analysis shows that the relative response was more strongly related to pore water
403 ionic strength than to soil or pore water Pb concentrations in most of the tests and in the
404 entire dataset (Table 4). Free ion Pb^{2+} activity poorly explained the toxicity (Table 4).
405
406 **Discussion**
407
408 A pronounced toxicity of Pb was observed in freshly spiked soils, with toxic thresholds (e.g.,
409 EC10 values) often near natural background levels of Pb in soil. In contrast, toxicity was
410 reduced greatly or even undetectable after 5 years of ageing outdoors under field conditions,
411 with the lowest EC10 = 350 mg Pb/kg soil for earthworms. Leaching, pH correction, and ageing
412 after spiking reduced toxicity by a factor of 8 (median value) based on EC10 values. Leaching
413 and pH corrections contributed to that shift in toxicity as experimentally shown here. The
414 factor f_2 , i.e. so-called ageing factor, is not only reflecting the ageing here because the
415 experimental leaching procedure adopted was not sufficient to remove excess salt in all soils.
416
417 The increased salinity in freshly spiked soils is likely the greatest modifying factor of toxicity as
418 suggested by the correlation analysis (Table 4), by the observation that the toxicity changes
419 were associated with the changes in reduced ionic strength across soils and treatments and
420 as suggested by literature data on salinity effects. Indeed, the critical salinity limit in pore
421 water for plant (tomato) growth is about 5 dS/m, about twice the limit for a saturated soil
422 extract (Marschner, 1995) which roughly corresponds to an IS of 65 mmol/L. The IS of freshly
423 spiked soils reached a maximum of 165 mmol/L and was still elevated at 125 mmol/L (soil TM)
424 and 75 mmol/L (soil BA) after the leaching protocol. Stevens et al. (2003) showed that soil Pb

425 toxicity thresholds (EC50) for growth of lettuce seedlings significantly increased by factors 2-
426 3 in three out of five soils after leaching due to the alleviation of the salinity stress. Leaching
427 also reduced chronic toxicity of $Pb(NO_3)_2$ in soil to *F. candida* by a factor of about 3 but no
428 such effect was found for $PbCl_2$ (Bongers et al., 2004). The lowest observed EC50 value in the
429 fully leached and aged soils was 1270 mg Pb/kg soil or about 5 mmol Pb/kg soil (Table 2).
430 Added as $PbCl_2$, this dose increases the pore water ionic strength to about 50 mmol/L, a level
431 at which salinity effects on plants begin to occur. Recently, a more intensive 10-day soil
432 spiking/leaching procedure was successful in removing most excess salt and acidification in
433 Cu^{2+} salt spiked soils (Schwertfeger and Hendershot, 2013a). This protocol might be tested for
434 Pb but needs to be scaled up for tests that require large quantities of soil such as earthworm
435 and plant tests.
436
437 The contribution of soil acidification to the adverse effects of Pb in spiked soils was first
438 observed for microbial processes (Speir et al., 1999). Although we observed decreases in soil
439 pH of up to 2 pH units in pore water, our experimental design does not allow isolation of
440 acidification effects because the soil liming was only conducted in the leached and
441 leached+aged soils, not in freshly spiked soils. The nitrification rate (PNR) is highly sensitive to
442 pH, and decreases by a factor of 2 between pH 7.0 and pH 5.5 (Smolders et al., 2001), indirectly
443 predicting that acidification might have contributed to the effect on nitrification, especially in
444 soil TM where soil pH decreased to 4.8. However, this soil pH is not sufficiently low to reduce
445 plant growth to a large extent and the effects of salinity would be more significant on Pb
446 toxicity to plants than soil acidification.
447 Although soil ageing effects on Pb toxicity (factor f_2) are somewhat larger than effects of
448 leaching/pH correction (factor f_1, Table 3), this must be interpreted with caution due to

449 incomplete removal of excess salt after leaching of soil TM and, to some extent, soil BA. Only
450 soil WB was sufficiently leached to reduce ionic strength so as to allow the quantification of
451 Pb toxicity reduction due to ageing. The ED10 values from earthworm reproduction and
452 substrate-induced respiration (SIR) tests provided ageing factors of 2.0 and 3.9, respectively
453 (Table 3). As discussed in the introduction, such ageing factors can also be estimated from soil
454 chemical analysis using the fraction of isotopically exchangeable metal in soil (Hamels et al.,
455 2014). Since the isotopically exchangeable fraction of Pb is rarely below 0.25, it appears
456 unlikely that ageing will alter toxicity by more than a factor of 4 (=1/0.25). A limited number
457 of studies have monitored the change in toxicity of Pb in unleached soils followed by toxicity
458 testing of the same soils after a period of ageing. A variety of microbial processes, including
459 SIR, were inhibited by Pb in freshly spiked soils with inhibitions less pronounced after a 3-
460 month ageing period (Zalaghi and Safari-Sinegani, 2014). Unfortunately, the low number of
461 doses (3) used precluded the estimation of valid ED10 values to calculate ageing factors, but
462 the limited data suggest a factor less than 4. The chronic toxicity of Pb to earthworms (*E.*
463 *fetida*) in an artificial soil decreased by a factor 1.6 after an 84-day ageing period (Chen et al.,
464 2014). The factors change in toxicity due to the 5 years ageing for the soil microbial processes
465 can also be ascribed to adaptation reactions since these population adapt along with ageing.
466 Lead tolerant microorganisms have indeed been identified in strongly Pb contaminated soils
467 (Baath et al., 2005).
468
469 Toxicity tests with plants (cowpea) in solution revealed that the EC50 is found near 1 µM
470 Pb^{2+}(Kopittke et al., 2011) . The Pb^{2+} (ion activity) in pore water was only above 1 µM in the
471 freshly spiked soil TM and was 0.1 µM or lower in aged soils suggesting that the significant
472 Pb^{2+} immobilization explains the relatively low ecotoxicity to plants in soils. In a previous study

473 (Cheyns et al., 2012), Pb toxicity to tomato plants was attributed to an indirect effect of P
474 deficiency in the shoots, probably caused by Pb phosphate precipitation in soil. The
475 aggregated dose response curve of the seven tests in three soils split according to the three
476 treatments after Pb^{2+} spiking, yielding an average (± standard error) EC50 of 2300 ±145 mg Pb/kg soil
477 in freshly spiked soils, 6500 ±750 mg Pb/kg soil in leached soils and >10,000 mg Pb/kg soil in leached
478 and 5 y aged soils.
479 Figure 5 shows a clear association between the plant P concentration and the yield of the plant
480 shoots after Pb spiking, again suggesting that the phytotoxic effects in all soils and soil
481 treatment may again be an indirect effects. Shoot P levels decreased with increasing soil Pb
482 for both tomato and barley shoots to levels below 0.20%, a value generally indicating P
483 deficiency (Marschner, 1995). In aged soils, Pb is likely less available for P precipitation
484 compared to freshly spiked soils, explaining the observed differences between Pb toxicity.
485 Soils were fertilized with P after the ageing period in order to allow ageing processes to effect
486 Pb immobilization and minimize the likelihood of Pb precipitation by added phosphate salts.
487
488 To conclude, the observed toxicity differences between freshly spiked soils and aged soils may
489 be explained by different factors. Salt stress is likely the most prominent modifying factor of
490 Pb toxicity in freshly spiked soils due to the strikingly high ionic strength in pore water and
491 because the acidification and ageing reactions are unlikely to explain the large magnitude of
492 the overall change in toxicity (i.e., the large f_3 factors). The overall statistical analysis of the
493 relative responses shows that ionic strength is better correlated with toxicity than total or
494 available Pb in soil (Table 4), corroborating that interpretation. The acute dosing of soluble
495 Pb^{2+} salts does not appear to be an appropriate model for environmental sources of Pb where
496 Pb gradually enters soils via atmospheric deposition as PbO, PbS, and $PbSO_4$ near smelters

(Sobanska et al., 1999) or via Pb in sewage sludge (i.e., Pb-phosphates and organically bound Pb). In order to reduce or avoid the confounding factors of toxicity resulting from the spiking of soils with Pb salts, it may be possible to use PbO fine powder as a source of Pb that does neither increase salinity nor acidifies soils. It has been shown that PbO is less toxic to plants than $PbCl_2$ when dose at equivalent Pb concentrations in soil but the kinetics of the PbO weathering reaction have not received attention (Khan and Frankland, 1983; Khan and Frankland, 1984). Alternatively, Pb-acetate has been uses as well in toxicity tests (Saint-Denis et al., 2001) which does not acidify the soil, but such compounds is not only not a relevant source of Pb and cannot be used for testing effects on microbial organisms since the substrate will likely stimulate microbial activity. Weathering rates of CdO (unpublished data) or ZnO have been found to be quite fast (~months) when evaluating relative to corresponding metal salts (e.g. (Smolders and Degryse, 2002) and it is worth investigating dissolution rates of PbO to better characterize the genuine toxic effects of Pb. In the absence of such data, results for Pb toxicity in soils freshly spiked with Pb salts must be corrected for the discrepancy with Pb toxicity observed under more environmentally relevant conditions.

Acknowledgement

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Tables

Table 1: Soil treatments and selected soil characteristics The total Pb concentration in the spiked soil is the range of measured concentrations across all spiking levels; other properties refer to the least contaminated soils.

Soil	treatment	Pb source	Organic C (g C/kg)	eCEC (cmolc/kg)	pH	Pb_{total} mg/kg	Location
BA	A: freshly spiked	$PbCl_2$	12	14.3	7.4	140-8700	Barcelona, Spain
	B: as A but leached and pH corrected	$PbCl_2$	14	14.7	7.4	140-7200	
	C: as B but aged	$Pb(NO_3)_2$	16	14.4	7.0	130-7000	
WB	A: freshly spiked	$PbCl_2$	43	26.5	6.1	52-6500	Woburn, UK
	B: as A but leached and pH corrected	$PbCl_2$	31	27.1	6.5	46-5000	
	C: as B but aged	$Pb(NO_3)_2$	33	22.3	6.7	100-5600	
TM	A: freshly spiked	$PbCl_2$	10	8.4	6.2	21-6600	Ter Munck, Belgium
	B: as A but leached and pH corrected	$PbCl_2$	10	8.7	6.7	22-7100	
	C: as B but aged	$Pb(NO_3)_2$	14	8.2	6.6	29-6400	

627 Table 2. The toxicity of Pb^{2+} salts in three soils for seven different tests as affected by the soil
628 treatment after spiking. The toxicity is expressed as the 50% effect concentration (EC50, total
629 soil concentration, measured). If the response curve could not be fitted or if the EC50 was >2-
630 fold above highest tested concentration, the EC50 is denoted as non-significant (n.s.)

		EC50 (mg Pb/kg soil-		
Soil	Test	A. freshly spiked	B. as A but leached and pH corrected	C as C but 5 y aged
BA	Tomato growth	2900	6370	12,600
	Barley growth	2380	7190	n.s.
	Nitrification rate (PNR)	3240	2200	n.s.
	Nitrification 28d (SIN)	7190	7120	n.s.
	Respiration (SIR)	8720	12,300	7020
	Eisenia fetida reprod.	480	1182	1270
	Folsomia candida reprod.	712	n.s.	n.s.
WB	Tomato growth	6140	6420	n.s.
	Barley growth	6750	5020	n.s.
	Nitrification rate (PNR)	2820	4920	n.s.
	Nitrification 28d (SIN)	1750	n.s.	n.s.
	Respiration (SIR)	9970	6160	n.s.
	Eisenia fetida reprod.	2400	1700	3280
	Folsomia candida reprod.	4530	5020	n.s.
TM	Tomato growth	1240	1430	4480
	Barley growth	1710	4580	n.s.
	Nitrification rate (PNR)	1470	1640	n.s.
	Nitrification 28d (SIN)	1410	2820	n.s.
	Respiration (SIR)	1680	8150	n.s.
	Folsomia candida reprod.	1710	2700 4256	n.s.

631

632 Table 3. The toxicity of Pb^{2+} salts in three soils for seven different tests as affected by the soil treatment after spiking. The toxicity is expressed as

633 the 10% effect concentration (total soil concentration, measured). The factor reduction in toxicity (f) due to soil treatment after spiking is the

634 factor change in EC10, calculated according to Eqns. (4) and (5). If the EC10 could not be estimated due to lack of toxicity, the factor reduction in

635 toxicity is > the ratio of the highest tested concentration to the corresponding EC10. All factors of reduced toxicity are significantly ($p<0.025$)

636 different from 1.0 except when noted as non-significant (n.s.).

		EC10 (mg Pb/kg soil)			factor reduction (f) in toxicity of *added Pb* at 10% effect		
Soil	Test	A. spiked	B. as A but leached and pH corrected	C. as B but 5 y aged	upon leaching and pH correction f_1=B/A	upon ageing f_2=C/B	upon leaching, pH correction and ageing f_3=C/A
BA	Tomato growth	730	3580	6480	2.7	1.9	12
	Barley growth	150	>7190	>7020	>540	n.d.	>530
	Nitrification rate (PNR)	960 695	330	>7020	0.7 n.s.	>35	>8.3
	Nitrification 28d (SIN)	4680 7500	4480	>7020	1.0 n.s.	>1.6	>1.5
	Respiration (SIR)	1580 6090	1852	1740 (573)	0.6 n.s.	2.3	1.8
	Eisenia fetida reprod.	190	560	350	1.9	1.1 n.s.	3.4
	Folsomia candida reprod.	320	438	>7020	12.8	>23.	>38
				median	**1.9**	**2.1**	**8.3**
WB	Tomato growth	2740	380	>5620	0.7	>16	>2.1
	Barley growth	2200	>5020	>5620	>2.3	n.d.	>2.6
	Nitrification rate (PNR)	720	1850	>5620	2.0	>3.1	>8.3
	Nitrification 28d (SIN)	700	1120	>5620	4.0	>5.1	>8.5
	Respiration (SIR)	110	1170	3730	5.3	3.9	65
	Eisenia fetida reprod.	1100	370	1610	0.7 n.s.	2.0	1.4 n.s.
	Folsomia candida reprod.	520	>5020	1660	>11	0.2 n.s.	2.7
				median	**2.3**	**3.5**	**2.7**

Soil	Test	EC10 (mg Pb/kg soil) A. spiked	B. as A but leached and pH corrected	C. as B but 5 y aged	factor reduction (f) in toxicity of *added Pb* at 10% effect: upon leaching and pH correction f_1=B/A	upon ageing f_2=C/B	upon leaching, pH correction and ageing f_3=C/A
TM	Tomato growth	460	160 (554)	420 (3210)	1.5	3.2	3.6
	Barley growth	320	710	5270	2.6	7.2	17
	Nitrification rate (PNR)	3401540	290	>6410	1.1 [n.s.]	>23	>20
	Nitrification 28d (SIN)	560	1800	5620	2.1	3.2	10
	Respiration (SIR)	190	670 (385)	1160	4.4	1.8 [n.s.]	7.9
	Folsomia candida reprod.	170	140 (1577)	>6410	1.5 [n.s.]	>55	>44
				median	**1.8**	**5.2**	**13**

637

638 Table 4. Correlation coefficients between the relative response and soil properties. All
639 correlations are significant at $p<0.05$, bold number are strongest correlation for each test.
640

Test	Total soil Pb concentration (mg/kg)	Pore water Pb (mg Pb/L)	Pb^{2+} ion activity (mol/L)	pH soil	Pore water ionic strength (mM)
Tomato growth	**-0.70**	-0.34	-0.32	0.31	-0.67
Barley growth	-0.27	-0.28	-0.27	0.40	**-0.40**
Nitrification rate (PNR)	-0.35	-0.26	-0.25	0.28	**-0.69**
Nitrification 28d (SIN)	-0.57	-0.36	-0.34	0.55	**-0.79**
Respiration (SIR)	-0.55	-0.43	-0.42	0.54	-0.58
Eisenia fetida reprod.	**-0.72**	-0.46	-0.31	-0.17	-0.46
Folsomia candida reprod.	-0.35	-0.18	-0.17	0.10	**-0.49**
All tests	*-0.42*	*-0.22*	*-0.21*	*0.20*	***-0.53***

641

642 **List of Figures**

643 Figure 1: Conceptual diagram showing the change in toxicity after spiking due to soil leaching and
644 ageing. The factors change in ED10 due to leaching and pH correction (f_1), ageing (f_2) and leaching+pH
645 correction + ageing (f_3) are illustrated. The diagram illustrates that the response curve of aged soils is
646 difficult to fit (see also Fig.2), therefore factors f were fitted on the combined data with Eqn. (5).

647 Figure 2: Pore water ionic strength (IS, mmol/L), soil pH and soil solution dissolved Pb concentrations
648 in the three soils as affected by the three treatments after spiking.

649 Figure 3: Selected dose-response curves illustrating the effects of soil leaching and 5 year ageing after
650 soil spiking with Pb^{2+} salts on Pb toxicity.

651 Figure 4: The aggregated dose response curve of the seven tests in three soils split according to the
652 three treatments after Pb^{2+} spiking, yielding an average (± standard error) EC50 of 2300 ±145 mg Pb/kg
653 soil in freshly spiked soils, 6500 ±750 mg Pb/kg soil in leached soils and >10,000 mg Pb/kg soil in
654 leached and 5 y aged soils.

655 Figure 5: The yield decline of tomato and barley in Pb^{2+} salt spiked soils is associated with a decrease
656 in shoot P concentration. A critical % P of 0.2 % is indicated with the dashed line. No Pb induced growth
657 decline is found at adequate P in the leaves (>0.30 % P). Filled symbols represent the freshly spiked
658 soils, empty symbols the spiked, leached and pH corrected soils and grey shaded symbols the spiked,
659 aged and pH corrected soils (circles: soil BA, triangles: soil WB and squares: soil TM).

660

661

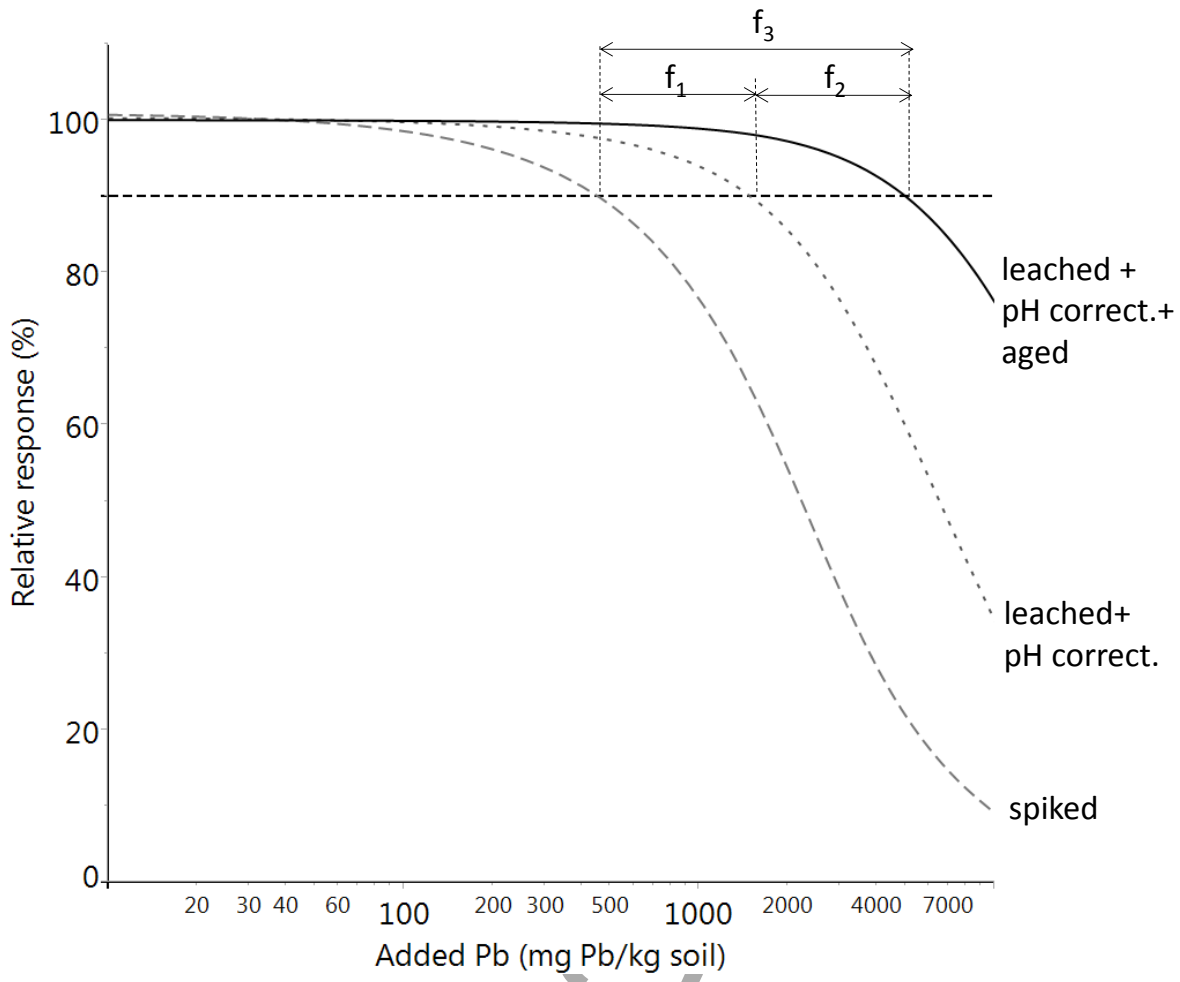
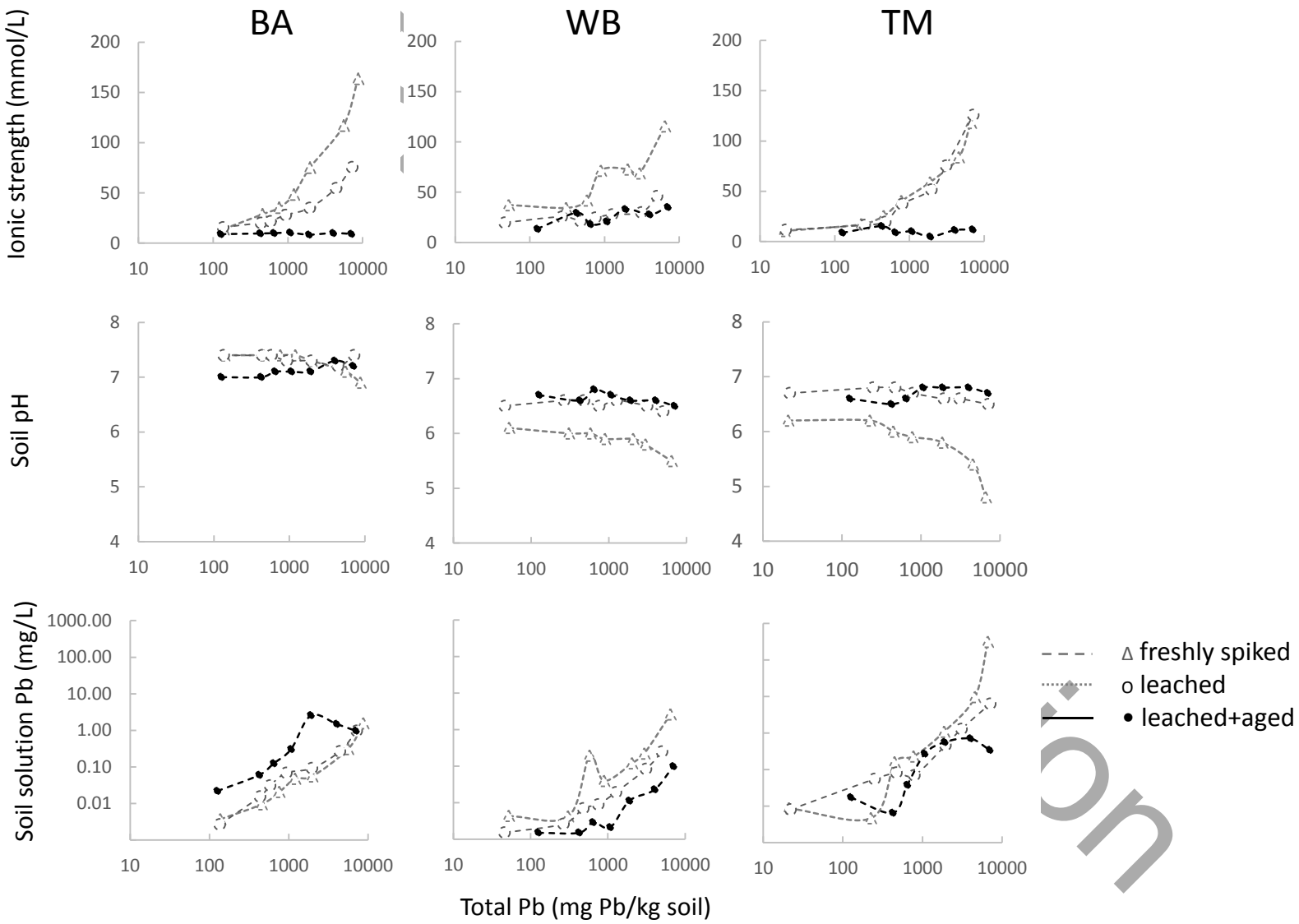


Figure 1.

Figure 2


BA
WB
TM
Ionic strength (mmol/L)
Soil pH
Soil solution Pb (mg/L)
Total Pb (mg Pb/kg soil)
freshly spiked
leached
leached+aged

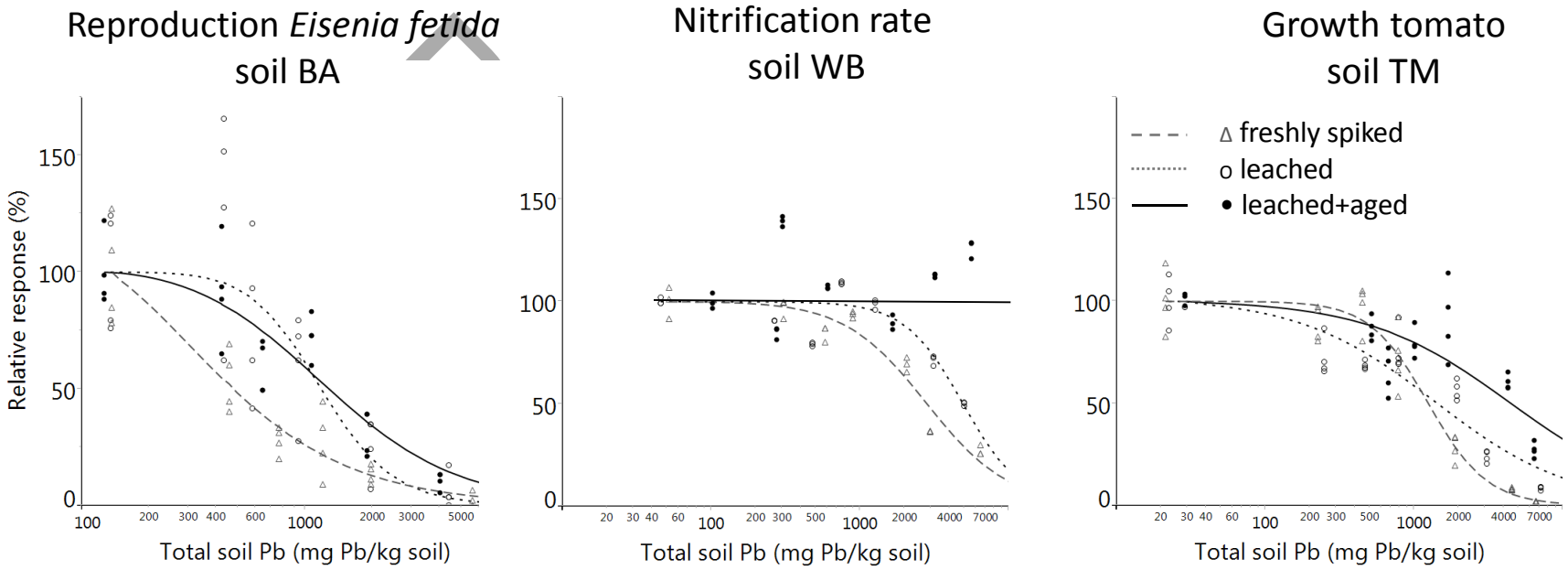


Figure 3.

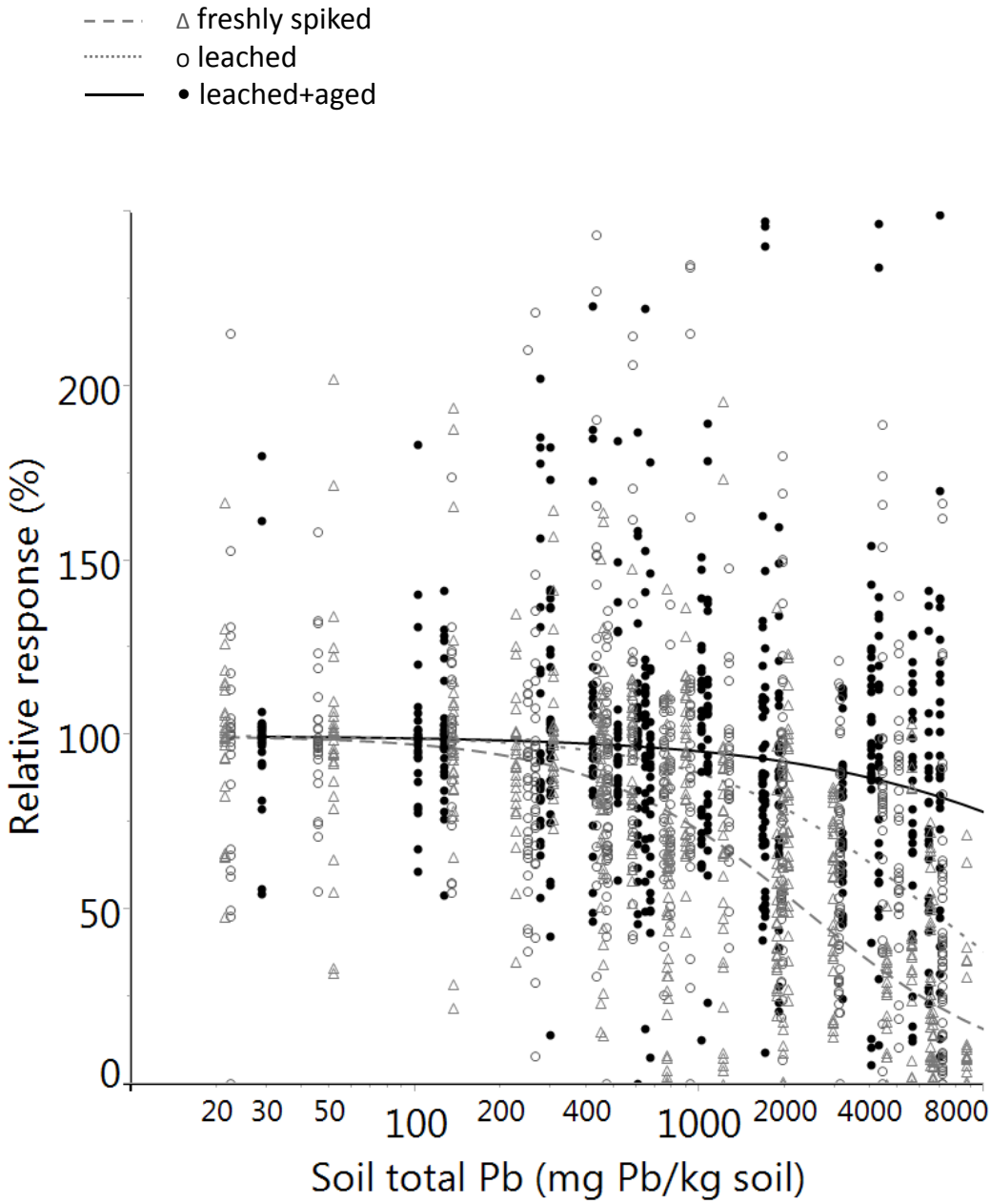

Δ freshly spiked
o leached
• leached+aged
Relative response (%)
0
50
100
150
200
20
30
50
100
200
400
1000
2000
4000
8000
Soil total Pb (mg Pb/kg soil)

Figure 4.

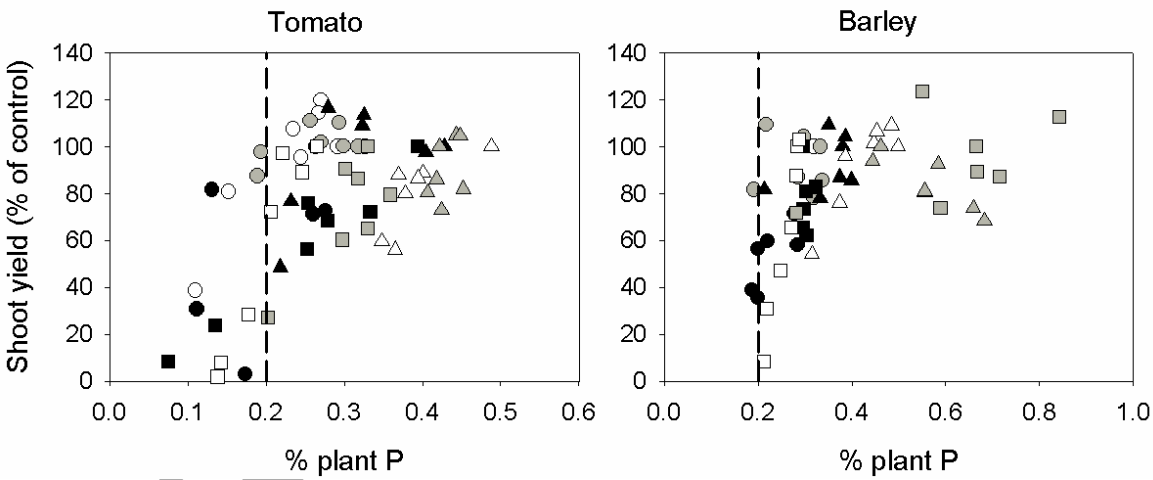


Figure 5.

680 Supporting information

681

682 Table SI1. The absolute response (mean, standard deviation, SD) the different biological assays for the
683 uncontaminated control soils as affected by soil (BA=Barcelona; TM= Ter Munck; WB = Woburn) and
684 treatment (SP=spiked; L=leached and pH corrected; A= leached, aged and pH corrected).

Test	Soil	Treatment	Mean	SD	n
Tomato shoot yield g dry matter/pot	BA	SP	0.51	0.08	4
	BA	L	0.41	0.06	4
	BA	A	0.87	0.03	4
	TM	SP	1.05	0.16	4
	TM	L	0.85	0.10	4
	TM	A	1.21	0.04	4
	WB	SP	1.05	0.04	4
	WB	L	0.97	0.04	4
	WB	A	0.87	0.05	4
Barley shoot yield g dry matter/pot	BA	SP	0.44	0.04	4
	BA	L	0.15	0.08	4
	BA	A	0.26	0.03	4
	TM	SP	0.38	0.09	4
	TM	L	0.47	0.12	4
	TM	A	0.28	0.01	4
	WB	SP	0.68	0.05	4
	WB	L	0.56	0.09	4
	WB	A	0.57	0.03	4
Potential nitrification rate (mg N/kg/day) (PNR)	BA	SP	9.1	0.4	3
	BA	L	12.4	0.4	3
	BA	A	8.6	0.1	3
	TM	SP	6.3	0.4	3
	TM	L	7.4	0.1	3
	TM	A	4.3	0.1	3
	WB	SP	5.2	0.4	3
	WB	L	7.6	0.1	3
	WB	A	10.3	0.4	3
Substrate induced nitrification (fraction of total added N, -) (SIN)	BA	SP	1.12	0.09	3
	BA	L	0.93	0.02	3
	BA	A	0.95	0.03	3
	TM	SP	1.12	0.01	3
	TM	L	1.17	0.06	3
	TM	A	1.14	0.01	3

Test	Soil	Treatment	Mean	SD	n
	WB	SP	0.73	0.29	3
	WB	L	1.10	0.04	3
	WB	A	1.14	0.02	3
Substrate induced respiration (fraction of glucose respired, -) (SIR)	BA	SP	0.30	0.01	3
	BA	L	0.25	0.01	3
	BA	A	0.31	0.01	3
	TM	SP	0.31	0.01	3
	TM	L	0.21	0.01	3
	TM	A	0.29	0.00	3
	WB	SP	0.24	0.01	3
	WB	L	0.26	0.01	3
	WB	A	0.30	0.01	3
Reproduction *E. fetida* hatchlings/container	BA	SP	45	10	4
	BA	L	29	8	4
	BA	A	39	6	4
	WB	SP	37	6	4
	WB	L	49	7	4
	WB	A	68	9	4
Reproduction *F. candida* hatchlings/container	BA	SP	693	429	10
	BA	L	1336	343	10
	BA	A	1102	327	10
	TM	SP	1729	622	10
	TM	L	1269	734	9
	TM	A	1411	579	10
	WB	SP	1547	861	10
	WB	L	1144	378	10
	WB	A	1070	417	10