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1 **Comparison of solid-phase bioassays and ecoscores to evaluate the toxicity**  
2 **of contaminated soils**

3

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15

16 **Abstract**

17

18 Five bioassays (inhibition of lettuce germination and growth, earthworm mortality,  
19 inhibition of springtail population growth, avoidance by springtails) were compared, using  
20 four coke factory soils contaminated by PAHs and trace elements, before and after bio-  
21 treatment. For each bioassay, several endpoints were combined in an 'ecoscore', a  
22 measure of test sensitivity. Ecoscores pooled over all tested bioassays revealed that most  
23 organisms were highly sensitive to the concentration of 3-ring PAHs. When four soils were  
24 combined, behavioural tests using the springtail *Folsomia candida* showed higher

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1 ecoscores, i.e. they were most sensitive to soil contamination. However, despite overall  
2 higher sensitivity of behavioural tests, which could be used for cheap and rapid  
3 assessment of soil toxicity, especially at low levels of contamination, some test endpoints  
4 were more sensitive than others, and this may differ from a soil to another, pointing to the  
5 need for a battery of bioassays when more itemized results are expected.

6

## 7 **Capsule**

8

9 The avoidance test using the soil springtail *Folsomia candida* is globally more sensitive to  
10 PAH contamination than acute and chronic toxicity bioassays using plants and animals  
11 but a battery of tests could reveal better in detail

12

13 *Keywords:* PAHs; Trace elements; Contaminated soils; Solid-phase bioassays; Toxicity  
14 tests; Avoidance tests; *Eisenia fetida*; *Lactuca sativa*; *Folsomia candida*; Ecoscores

15

## 16 **1. Introduction**

17

18 Industrial activities lead to the discharge of a wide range of hazardous chemicals in  
19 soils, often far from emission sources (Jones et al., 1989; Nam et al., 2008). Soil  
20 pollutants include polycyclic aromatic hydrocarbons (PAHs) and heavy metals, known for  
21 potential adverse ecological and toxicological effects (Bispo et al., 1999; Peralta-Videa et  
22 al., 2002; Boularbah et al., 2006). Polluted soils also are a threat to ecosystem and human  
23 health (Menzie, 1992; Lawlor et al., 1997; Preuss et al., 2003). This threat is generally  
24 approached by quantifying the total content of pollutants in the contaminated matrices.  
25 Nevertheless this provides only limited information on pollutant bioavailability, and no  
26 information on synergetic or antagonistic interactions between pollutants (Juvonen et al.,  
27 2000), or on effects on organisms, for which only a biological approach is effective

1 (Fernández et al., 2005). An ecotoxicological approach, using biological tests on target  
2 organisms at different trophic levels, has been recommended for a refined evaluation of  
3 environmental hazards in complement of chemical analyses (Bispo et al., 1999; Rila and  
4 Eisentraeger, 2003; Fernández et al., 2005; Plaza et al., 2005). Indeed, bioassays  
5 integrate the impact of all contaminants including those not considered or detected by  
6 chemical analyses, and they take account of additive, synergistic and antagonistic  
7 phenomena.

8

9         Direct toxic effects on survival, growth or reproduction of test organisms may  
10 reflect the ecotoxicological potential of contaminated soils (Fent, 2003). Phytotoxicity  
11 tests, such as lettuce bioassays, provide a variety of assessment endpoints such as  
12 germination and root elongation rates and enzyme activities (Ferrari et al., 1999). Soil  
13 invertebrates have also been used in ecotoxicology, in particular earthworms (Fernández  
14 et al., 2005; Eom et al., 2007), enchytraeids (Römbke, 2003), springtails (Domene et al.,  
15 2007; Eom et al., 2007) and woodlice (Jänsch et al., 2005; Loureiro et al., 2005).  
16 Springtail, earthworm and lettuce soil quality tests have been standardized according to  
17 ISO (1999), ISO (1993a, 1998a) and ISO (1993b, 2005a), respectively.

18

19         Based on the ability of animals to probe and flee from contaminated places (Best  
20 et al., 1978; Salminen and Sulkava, 1996; Gass et al., 2006), avoidance tests have a  
21 great potential as early screening tools in lower tier levels of ecological risk assessment,  
22 because they are robust, sensitive, cost-effective, ecologically relevant and rapid (Natal-  
23 da-Luz et al., 2004, 2008a). Avoidance tests are now currently performed with earthworms  
24 (Loureiro et al., 2005; Natal-da-Luz et al., 2008a, b; Garcia et al., 2008; De Silva and Van  
25 Gestel, 2009; Owojori and Reinecke, 2009), enchytraeids (Amorim et al., 2008; Loureiro  
26 et al., 2009; Kobetičová et al., 2009), woodlice (Loureiro et al., 2009) and springtails  
27 (Heupel, 2002; Martínez Aldaya et al., 2006; Natal-da-Luz et al., 2008a, b, 2009) and an

1 international standard for the assessment of soil quality using earthworm avoidance tests  
2 exists (ISO, 2008a).

3

4         Several studies compared some toxicity and avoidance endpoints (Greenslade  
5 and Vaughan, 2003; Loureiro et al., 2005; Martínez Aldaya et al., 2006), but a comparison  
6 between tests commonly used for the biological assessment of soil quality is clearly  
7 lacking, and studies using a battery of soil and aquatic test organisms did not include  
8 avoidance endpoints (Fernández et al., 2005; Pandard et al., 2006; Domene et al., 2008).  
9 Such a comparison should be valid both for scaling toxicity and behavioural tests  
10 according to their sensitivity as early screening tools, and for pooling them in a bulk index  
11 of soil toxicity.

12

13         The reported work evaluates the toxicity of contaminated soils by comparing a  
14 variety of solid-phase bioassays applied to PAH-contaminated soils issued from former  
15 coke sites in northern France. Studied soils differed by their PAH content and the  
16 presence or not of a mixed pollution by heavy metals and/or cyanides. The aims of our  
17 study were: (1) to characterize contaminated soils using ecotoxicological (including  
18 behavioural endpoints) and chemical analyses, (2) to estimate the likely relationships  
19 between pollutants and toxicity responses, (3) to compare the sensitivity of toxicity tests  
20 representing different trophic and toxicity levels with the *Folsomia candida* (Collembola)  
21 avoidance test. Toxicity tests relied on the germination and growth of the lettuce *Lactuca*  
22 *sativa* (Asteraceae) and on the survival and reproduction of the springtail *F. candida*  
23 (Isotomidae) and the earthworm *Eisenia fetida* (Lumbricidae). Two alternative hypotheses  
24 were (1) either a test or a group of tests gives a better response to all soils and thus could  
25 be used preferentially as a sensitive indicator of soil quality, its performance being  
26 measured by an 'ecoscore', or (2) each test or group of tests exhibits a specific response

1 and as a consequence is not enough to assess soil quality, in which case several  
2 bioassays are necessary.

3

## 4 **2. Materials and methods**

5

### 6 *2.1. Soil samples*

7

8 Experiments were carried out on PAH-contaminated soils from three industrial  
9 sites located in the North of France, the main activity of which was the distillation of coal  
10 tar. Soil 1 was fairly polluted with a mixture of PAHs, cyanides and heavy metals. Soil 2  
11 was recovered after 18 months of windrow biotreatment. Despite bioremediation, this soil  
12 was still characterized by a high content of PAHs, cyanides and heavy metals. Contrary to  
13 Soil 2, Soil 3 was only polluted by PAHs, with a concentration similar to that of Soil 2. In  
14 the same site a windrow biotreatment was applied to this soil and Soil 3T was sampled  
15 after six months of biotreatment (Lors et al., 2009). After biotreatment, Soil 3T showed a  
16 PAHs concentration lower than that of Soil 1.

17

18 Unpolluted soils were also sampled in the three studied sites in uncontaminated  
19 areas (Table 2), which were used as controls in the avoidance test and as a matrix of  
20 dilution in toxicity bioassays. Previous chemical and ecotoxicological analyses were  
21 performed on control soils, which did not reveal any toxicity.

22

### 23 *2.2. Chemical analyses*

24

25 Soil pH<sub>water</sub> was measured using a Consort<sup>®</sup> C83 pH-meter fitted with a glass  
26 electrode corrected for temperature and a Schott<sup>®</sup> box with Ingold<sup>®</sup> combined electrodes,  
27 according to ISO (2005b). Total organic carbon concentration was obtained from total

1 carbon and inorganic carbon contents, determined with a TOC-5000A Shimadzu®  
2 analyser, according to ISO (1995a). Total organic nitrogen concentration was determined  
3 by the Kjeldahl method, according to ISO (1995b). Total phosphorus as well as metals  
4 (As, Cd, Co, Cr, Cu, Ni, Pb, Zn) were dosed by Inductive Coupled Plasma Atomic  
5 Emission Spectrometry (ICP-AES) in a 138 Ultrace Jobin Yvon® analyser after hot  
6 hydrofluoric and perchloric acid digestion of solid phase, according to ISO (2001, 2008b).

7  
8 Concentrations of the 16 PAHs of the US-EPA list (Verschueren, 2001) were  
9 measured using High Performance Liquid Chromatography (HPLC) in a 2690 HPLC  
10 Waters® analyser fitted with an ultraviolet inverted phase C 18 Supelco® column (length  
11 250mm, internal diameter 2.1m) coupled to a 996 Waters® UV photodiode array detector  
12 according to ISO (1998b), after extraction by dichloromethane/acetone (50/50 v/v) using  
13 the Accelerated Solvent Extractor Dionex® ASE 200. Total cyanides were determined  
14 according to ISO (2003). All chemical analyses were done in triplicate.

15

### 16 2.3. Toxicological analyses

17

18 Toxicity results were the responses of test organisms according to concentration of  
19 soil samples in test media (% w/w). NOEC was the highest effective concentration at  
20 which no significant effect was detected, while EC<sub>10</sub>, EC<sub>20</sub> and EC<sub>50</sub> were the calculated  
21 concentrations at which the measured endpoint was reduced to 10%, 20% and 50% of the  
22 control value, respectively. Toxic effects were also calculated as percent inhibition at the  
23 highest concentration of the contaminated soil and as Toxic Units or TU (= 100/ EC<sub>50</sub>). In  
24 mortality tests (endpoint survival), results were expressed as lethal concentrations  
25 reducing survival by 10%, 20% and 50% (LC<sub>10</sub>, LC<sub>20</sub> and LC<sub>50</sub>, respectively) compared to  
26 controls.

27

1           Phytotoxicity tests were conducted according to ISO (1993b, 2005a), using only *L.*  
2 *sativa* (lettuce). Tests were carried out in a chamber at  $20\pm 2^{\circ}\text{C}$  under constant illumination  
3 (4000–7000 lx), with a 16:8 day-night light cycle. Assays were conducted in plastic pots  
4 (diameter 11cm, height 10cm) containing 200 g of contaminated substrate moistened at  
5 70-80% water-holding capacity. The moisture level was maintained constant by adding  
6 distilled water every day. Twenty seeds were placed at the surface of the test medium.  
7 Five concentrations of the contaminated soil were tested: 100%, 60%, 35%, 20% and  
8 10%, w/w. For each concentration, analyses were done in triplicate. Seedling emergence  
9 (%) was determined after seven days of exposure. Seedling wet and dry biomasses were  
10 measured after 14 days of exposure. Results were expressed as percent lettuce  
11 germination and growth in comparison with controls.

13           Acute toxicity tests with the earthworm *E. fetida* were carried out according to ISO  
14 (1993a). The survival of adult earthworms was determined after 14 days of exposure. Ten  
15 individuals were placed in a glass jar containing 500g of wet soil at 70-80% (w/w)  
16 moisture. Various concentrations of the studied soil in the control soil were tested in the  
17 range 1–100 %. For each tested concentration, four replicate cultures were done. The jars  
18 were exposed in an environmental chamber at  $20\pm 1^{\circ}\text{C}$  under a 16:8 (400 – 800 lx) day-  
19 night light cycle. Results were expressed as percent mortality in comparison with controls.

21           The springtail reproduction test was conducted according to ISO (1999), modified  
22 according to Martínez Aldaya et al. (2006). Population growth responses were assessed  
23 by introducing 10 parthenogenetic females of *F. candida* into each of five rearing  
24 chambers (crystal polystyrene boxes 45mm diameter, 25mm height), fifth-filled with the  
25 control soil or with the polluted soil at 0.35%, 1%, 5%, 10%, 50% and 100% concentration.  
26 A small amount of dry cattle dung powder was added above the soil substrate before  
27 animals were introduced, then boxes were incubated at  $20^{\circ}\text{C}$  in darkness during 40 days.

1 At the end of the experiment, the whole population was collected, using forceps and  
2 flotation.

3

4 Avoidance tests were conducted according to Martínez Aldaya et al. (2006) and  
5 Lors et al. (2006). They were performed in sterile crystal polystyrene Petri dishes (55mm  
6 diameter, 10mm height), the bottom of which was lined with two half-disks of glass-fibre  
7 filter paper (50mm diameter). The entire surface of each half-disk was covered with a  
8 paste of soil which was prepared just beforehand by adding enough deionized water to  
9 make the soil plastic. One half-disk was covered with the control soil, the other with the  
10 polluted soil diluted at the same concentrations as for population growth experiments. The  
11 two half-disks were separated by a 2mm space line, at the centre of which a single  
12 individual of *F. candida* was deposited. Animals selected for avoidance tests were naive  
13 adults or subadults and came from the same batch culture originating from a single female  
14 collected in the Park of the Brunoy Laboratory. Batch cultures had been maintained on  
15 fine quartz sand with ground cow dung as food for more than two years. The position of  
16 the animal was recorded each 20 min up to 100 min. Twenty replicates, in two successive  
17 batches of ten, were followed together under a Sharp® fluorescent illuminator in a  
18 chamber at 20°C. The position of the animal was checked through the cover lid by help of  
19 a hand-held magnifying glass. Blank experiments did not detect any light gradient which  
20 could bias the results (Salmon and Ponge, 1998). Totals of five counts over 100 min for  
21 each Petri dish were used as scores for testing differences between control and polluted  
22 sides.

23

24 Toxic effects were calculated as percentages of inhibition at a given concentration  
25 or as  $LE_{Cx}$  values. Percent inhibition was determined with respect to the control soil.  $LE_{Cx}$   
26 values were calculated following adjustment of data to a log-probit logistic model  
27 (Litchfield and Wilcoxon, 1949). NOEC was the highest concentration tested that did not

1 significantly differ from control at 5% risk level. LOEC was not used and was replaced by  
2 EC<sub>10</sub> or LC<sub>10</sub>. Toxicity values were also expressed into Toxic Units (TU), using the formula  
3  $TU = 100/EC(\text{or } LC)_{50}$ .

4

5 From five ecotoxicological parameters E(L)C<sub>50</sub>, E(L)C<sub>20</sub>, E(L)C<sub>10</sub>, NOEC, and %  
6 inhibition, 'ecoscores' were calculated by giving to each value a score between 0 and 3 as  
7 a function of its intensity, according to the following scales (x = endpoint value):

8 • for E(L)C<sub>50</sub>, E(L)C<sub>20</sub>, E(L)C<sub>10</sub>, and NOEC

9 ○ 0 = no effect ( $x > 100$ )

10 ○ 1 = weak effect ( $50 < x \leq 100$ )

11 ○ 2 = medium effect ( $20 < x \leq 50$ )

12 ○ 3 = strong effect ( $x \leq 20$ )

13 • for % inhibition

14 ○ 0 = no effect ( $x \leq 5$ )

15 ○ 1 = weak effect ( $5 < x \leq 20$ )

16 ○ 2 = medium effect ( $20 < x \leq 60$ )

17 ○ 3 = strong effect ( $x > 60$ )

18 Then the five unit scores were summed up and the total was rescaled to 100 for maximum  
19 intensity of the five endpoints.

20

21 Correlation analysis, using Pearson product-moment correlation coefficient, was  
22 used to explore possible linear relationships between physicochemical parameters and  
23 toxicity endpoints. All calculations were done with the statistical software XSTAT<sup>®</sup>  
24 (Addinsoft, Paris, France) using Excel<sup>®</sup> (Microsoft, Redmond, WA).

25

### 26 3. Results

27

### 1 3.1. Chemical data

2

3 Physicochemical characteristics of studied soils are reported in Table 1. Moisture  
4 content was close to 20% (w/w) in Soils 2, 3 and 3T whereas it was only 10% in Soil 1. All  
5 soils had a pH value close to 8 and most of their carbon was organic. However, Soil 2  
6 presented a much higher amount of organic carbon (44%) compared to Soils 1 and 3 (<  
7 10%). This difference could be explained by the addition of compost during windrow  
8 treatment of Soil 2, as confirmed by high N and P amounts. Concentrations of PAHs and  
9 trace elements (cyanides and heavy metals) in tested and control soils are presented in  
10 Table 2 and the 16 PAHs of the US-EPA list are detailed in Table 3 for the four tested  
11 soils. Concentrations of trace elements were compared to the geochemical background in  
12 the North of France (Sterckeman et al., 2002).

13

14 Soil 3 was mainly contaminated by organics, since heavy metals and cyanides  
15 were present in low concentrations (Table 2). Concentrations of heavy metals were close  
16 to the geochemical background, to the exception of slightly more Zn. Cyanides did not  
17 exceed 1 mg.kg<sup>-1</sup>. Soil 3 was heavily polluted with PAHs, with a global content of 3g.kg<sup>-1</sup>  
18 dry soil (Table 2). Most PAHs were 2-, 3- and 4-ring compounds (Table 3), 3-ring PAHs  
19 being dominant (44% of  $\Sigma 16$  PAHs), followed by 2- and 4-ring compounds, which  
20 amounted to 28 and 20 % of  $\Sigma 16$  PAHs, respectively (Fig. 1). Among 3-ring PAHs,  
21 phenanthrene was dominant (49%), followed by acenaphtene, fluorene and anthracene in  
22 similar concentrations (17, 16 and 18%, respectively). Fluoranthene was the main 4-ring  
23 PAH (51%), followed by pyrene (29%). The 5- and 6-ring PAHs were hardly represented  
24 in Soil 3, amounting to 5 and 2 % of  $\Sigma 16$  PAHs, respectively (Fig. 1).

25

26 Soil 3T, i.e. Soil 3 after six months of windrow biotreatment, showed a much lower  
27 PAH concentration, roughly one tenth that of untreated soil (Table 1). Biotreatment led to

1 a strong degradation of 2-, 3- and 4-ring PAHs, which decreased by 98, 97 and 82%,  
2 respectively (Fig. 1). Among the remaining compounds, 4-ring PAHs were most  
3 represented (42% of  $\Sigma 16$  PAHs), amounting to  $146 \text{ mg.kg}^{-1}$  dry soil. As expected, the  
4 concentration of total trace elements did not decrease nor increase after biotreatment.

5  
6 Contrary to Soil 3, Soil 2 showed a dual organic and inorganic contamination. Its  
7 total content of Zn, Pb, Cu and Cd was close to  $1 \text{ g.kg}^{-1}$  dry soil, i.e. 8 times the  
8 geochemical background (Table 2). Cyanides were in considerable amount in Soil 2,  
9 about  $70 \text{ mg.kg}^{-1}$  dry soil. This soil was also highly polluted in PAHs (Table 2), to the same  
10 level as Soil 3 ( $\Sigma 16$  PAHs =  $3.69 \text{ g.kg}^{-1}$  dry soil). Despite similar global amounts of PAHs  
11 in Soils 2 and 3, their distribution was different (Fig. 1). Soil 2 was dominated by 4-ring  
12 compounds, which amounted to 50% of  $\Sigma 16$  PAHs ( $1727 \text{ mg.kg}^{-1}$  dry soil), with  
13 fluoranthene as the main 4-ring compound (36%), followed by pyrene,  
14 benzo[a]anthracene and chrysene (Table 3). The 3-ring PAHs represented only 17% of  
15  $\Sigma 16$  PAHs ( $624 \text{ mg.kg}^{-1}$  dry soil), probably due to partial degradation during windrowing.  
16 The predominance of phenanthrene (49%) was also noticeable along with a smaller  
17 proportion of anthracene (33%). The 5- and 6-ring PAHs were also present in higher  
18 amounts in Soil 2 than in Soil 3 (Fig. 1). Globally, Soil 2 was thus polluted by PAHs of  
19 higher molecular weight than Soil 3.

20  
21 Soil 1 also presented a mixed pollution (Table 2). However, PAHs, cyanides and  
22 heavy metals were in lower amounts than in Soil 2. The concentration of PAHs was about  
23  $1 \text{ g.kg}^{-1}$  dry soil, with 3- and 4-ring PAHs most represented, amounting each to 40% of  
24  $\Sigma 16$  PAHs, i.e. around  $300 \text{ mg.kg}^{-1}$  dry soil (Fig. 1). Anthracene, phenanthrene and  
25 fluoranthene were the main 3- and 4-ring compounds (Table 3). Within heavy metals, only  
26 Cu and As showed contents about three times the geochemical background. Cyanide  
27 concentration of Soil 1 was ten times less than in Soil 2.

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### 3.2. Acute and chronic toxicity endpoints

Lettuce germination bioassays indicated that Soil 3 was highly toxic to seeds (ecoscore 93%), and that it lost totally its toxicity after 6-month biotreatment (ecoscore 0%). Soils 1 and 2 exhibited about half the toxicity of Soil 3, as ascertained by their ecoscore (47% each). The germination of lettuce seeds was inhibited by 71% in Soil 3, compared to about 20% in Soils 1 and 2 (Table 4). No inhibition of germination was observed in Soil 3T. It must be noticed that ecoscores equalized differences between Soils 1 and 2 when comparing  $EC_{20}$  and  $EC_{50}$  separately. Similarly, the growth of *L. sativa* was strongly inhibited by Soil 3 (80%), while Soils 1 and 2 inhibited it to a weak extent (0.7 and 10%, respectively). Ecoscores embraced the inhibition of growth at the highest concentration tested and weaker toxic effects which could be observed at lower concentration (Table 5). The ecoscore for growth inhibition amounted to 93% for Soil 3 (same value as for germination inhibition), while it remained low for Soils 1 and 2 (13% and 20%, respectively). After biotreatment, Soil 3 totally lost its toxic effects towards lettuce growth. Thus, according to the two lettuce endpoints, Soil 3 appeared highly toxic (acute toxicity), while Soils 1 and 2 had a moderate effect on this organism. According to ecoscores, lettuce germination appeared more sensitive than lettuce growth.

Similarly to lettuce bioassays, the earthworm mortality bioassay (acute toxicity) showed that Soil 3 was highly toxic (inhibition rate 100%, ecoscore 100%), but contrary to lettuce germination and growth inhibition, Soil 1 did not affect at all the survival of *E. fetida* (inhibition 0%, ecoscore 0%). Soil 2 was at an intermediate level and Soil 3 after biotreatment (Soil 3T) did not reveal any residual toxicity (Table 6).

1           In the springtail population growth test (chronic toxicity), Soil 3 appeared highly  
2 toxic, as in lettuce and earthworm acute toxicity tests, with an inhibition rate and an  
3 ecoscore of 100% (Table 7). According to raw data (not shown), acute toxicity of Soil 3  
4 was exemplified by the total disappearance of the population of *F. candida* within the  
5 duration of the experiment, even when this soil was diluted at 5%. Soils 1 and 2 were not  
6 classified in the same manner as in lettuce and earthworm bioassays. Soil 2 did not elicit  
7 any response by *F. candida* despite its pollution (inhibition rate and ecoscore 0%), while  
8 Soil 1 inhibited partially its population growth (inhibition 64%, ecoscore 60%). Contrary to  
9 other toxicity bioassays, a weak but significant response of population growth was  
10 detected after 6-month biotreatment in Soil 3T. The EC<sub>10</sub> value indicated that 10% of the  
11 full response was obtained at 5% concentration of the contaminated soil, and the inhibition  
12 rate was 27% when *F. candida* was grown on the pure soil (highest concentration used in  
13 the experiment). This resulted in an ecoscore of 53%, quite different from the ecoscore of  
14 0% obtained with the other three bioassays.

15

### 16 3.3. Behavioural endpoint

17

18           According to ecoscores and inhibition rates, Soil 3 (before biotreatment) appeared  
19 highly repellent (ecoscore 100%, inhibition rate 100%). Soils 1 and 2 were also repellent,  
20 although at a lower level (ecoscores 80% and 67%, respectively), while the inhibition rate  
21 (of the pure soil) was moderate (9.5% and 12.5%, respectively). This discrepancy can be  
22 explained by very low EC<sub>20</sub> and EC<sub>10</sub> values (Table 8), indicating that the avoidance test  
23 was very sensitive at the highest level of dilution (0.35%). The repellence of Soil 3 totally  
24 disappeared after 6-month biotreatment (Soil 3T), while as mentioned above population  
25 growth bioassays still detected some marginal toxicity in this soil.

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### 27 3.4. Ecoscores

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A global comparison of ecoscores for the five toxicity and behavioural endpoints (Table 9) showed that lettuce germination and springtail avoidance classified Soil 3 (high content in low molecular weight PAHs) as the most highly threatened soil, while Soils 1 and 2 (mixed pollution) exhibited a lower level of toxicity. The toxicity of Soil 3T was not detected by these bioassays after 6-month biotreatment. However, the toxicity of Soils 1, 2 and 3, as measured by ecoscores, was higher with to the springtail avoidance test (80%, 67% and 100%, respectively) than with the lettuce germination test (47%, 47% and 93%, respectively), indicating a better sensitivity of behavioural endpoints. Lettuce growth appeared less sensitive to pollution of Soils 1 and 2 than germination. Earthworm mortality and springtail reproduction tests depicted the same high level of toxicity for Soil 3. However, the toxicity of Soil 2 was revealed by the former and that of Soil 1 by the latter bioassay, which also revealed a marginal toxicity in Soil 3T. By pooling ecoscores for the four soils, the five bioassays can be classified in an increasing order of sensitivity (decreasing ecoscores) according to springtail avoidance > springtail reproduction > lettuce germination > earthworm mortality > lettuce growth (Table 9).

By pooling ecoscores for the five bioassays, the four soils can be classified in an increasing order of toxicity (increasing ecoscores) according to Soil 3T << Soil 2 < Soil 1 << Soil 3 (Table 9). Among the different PAHs of the US-EPA list, 3-ring PAHs were those to which most tested bioassays were sensitive. Ecoscores pooled over the five bioassays showed a positive and significant relationship with the concentration of 3-ring PAHs, which explained 90% of their total variation (Fig. 2). No significant relationship was observed with trace elements.

#### **4. Discussion**

1 4.1. Different soils may display different responses in toxicity and behavioural laboratory  
2 tests

3

4 Lors et al. (2006) showed that avoidance by *F. candida* of a coke factory soil  
5 (diluted to 1% in a control soil) disappeared after 2-month incubation at 20°C and that this  
6 was accompanied by a pronounced diminution of 3- and 4-ring PAHs. All tests performed  
7 on Soils 3 and 3T (before and after 6-month windrow biotreatment) showed that the  
8 untreated soil was highly acute and chronic toxic (all bioassays responded in the same  
9 way, with ecoscores near or equal to 100) and lost all or part of its toxicity after  
10 bioremediation (Table 9). However, it should be noticed that the growth of *F. candida*  
11 population was still negatively affected, although to a weak extent, after 6-month  
12 biotreatment (Table 7). We suspect that the dissipation of PAHs in Soil 3 was incomplete,  
13 and that either recalcitrant (high-molecular weight) PAHs were still present (Smith et al.,  
14 1999), most probably associated with added organic matter in soil aggregates (Amellal et  
15 al., 2001; Nam and Kim, 2002; Nam et al., 2008), or low-molecular weight PAHs were still  
16 active at low doses against some sensitive soil fauna (Johnson et al., 2002). The  
17 particular toxicity of 3-ring PAHs has been demonstrated at the biomolecular level by  
18 Incardona et al. (2005) and was confirmed by our toxicity endpoints.

19

20 Soils 1 and 2 (mixed pollution, with a high content of cyanides in Soil 2) displayed  
21 some toxicity, although less than Soil 3, which was detected or not by tested bioassays,  
22 according to soil (Table 9). Lettuce and avoidance bioassays detected the toxicity of both  
23 soils, while that of Soil 1 was detected by *F. candida* and not by *E. fetida* toxicity tests.  
24 The contrary was observed for Soil 2, which exhibited acute toxicity to *E. fetida* but no  
25 chronic toxicity to *F. candida*. These results point to differential sensitivity of test  
26 organisms according to nature of pollution. Differences between woodlice and earthworms  
27 were clearly assessed in a comparative study by Loureiro et al. (2005), who showed that

1 according to the nature of contamination they did not respond in the same manner. This  
2 urged ecotoxicologists to turn to battery tests using a variety of organisms pertaining to  
3 different trophic levels (Pandard et al., 2006; Domene et al., 2008) or to multispecies soil  
4 systems (Fernández et al., 2005).

5

#### 6 4.2. The choice of a strategy for the laboratory assessment of environmental hazards

7

8 We showed that over the whole array of studied soils, toxicity and behavioural  
9 tests could be compared by their ecoscores, a bulk assessment of total and partial  
10 inhibition of biological endpoints, in place or in addition to particular parameters (NOEC,  
11 LOEC, EC<sub>50</sub> or percent inhibition at the highest concentration) which do not embrace the  
12 whole biological response. This method allowed us to classify the avoidance test using *F.*  
13 *candida* as more sensitive than the corresponding reproduction test, and both tests as  
14 superior to earthworm and plant bioassays. Similarly, Eom et al. (2007) showed that the *F.*  
15 *candida* reproduction test was more sensitive than earthworm (*E. fetida*) and plant (*L.*  
16 *sativa* and *Brassica chinensis*) tests to PAH soil contamination, but they did not compare  
17 them with behavioural tests. Martínez Aldaya et al. (2006) showed that avoidance and  
18 reproduction tests using *F. candida* were equally sensitive to coke factory soils but their  
19 dose-response curves seem to indicate that behavioural responses were more  
20 progressive than toxicity responses, the latter exhibiting clear threshold effects. This might  
21 be compared with our avoidance EC<sub>20</sub> and EC<sub>10</sub> values, which were very low (Table 8)  
22 compared to other tests (Tables 4-7). This, together with the higher sensitivity of  
23 behavioural tests as estimated by ecoscores (Table 9), suggests that avoidance tests  
24 could be preferred when a finer dose-response relationship is requested. Natal-da-Luz et  
25 al. (2008b) showed that earthworms (*Eisenia andrei*) were more sensitive than Collembola  
26 (*F. candida*) to soil texture and thus the latter test organism should be preferred when a  
27 large array of soils of different provenances are to be compared. The rapid and stable

1 response of springtail behavioural tests is remarkable, and we suggest decreasing the  
2 exposure time of 24 h proposed by Natal-da-Luz et al. (2008a) to only 1-2 h, on the base  
3 of present study and knowledge on springtail choice ability (Sadaka-Laulan et al., 1998;  
4 Salmon and Ponge, 2001; Boitaud et al., 2006).

5  
6 If behavioural tests using soil invertebrates seem justified from an ecological point  
7 of view (Yearley et al., 1996), main criticism lies on whether soil invertebrates are able to  
8 perceive pollutants and to escape them before being affected. In case an odour can be  
9 detected through the emission of volatiles, which is the case for low molecular weight  
10 PAHs, the avoidance test can be efficient and rapid, as most soil animals live in darkness  
11 and use odours as clues when foraging (Hedlund et al., 1995; Salmon and Ponge, 2001).  
12 The only exception is when mortality is immediate or animals are totally or partly  
13 paralysed due to neurotoxic effects of PAHs (Ritchie et al., 2001; Preuss et al., 2003;  
14 Martínez Aldaya et al., 2006). Martínez Aldaya et al. (2006) showed that this is a question  
15 of dosage, since repellence occurs at very low doses, far below acute toxicity levels.

16  
17 To the light of our results and after screening published literature, a double  
18 strategy could be suggested. Behavioural tests should be preferred to toxicity tests as  
19 early assessment tools of soil pollution by PAHs when the level of pollution is expected to  
20 be feeble or when nothing is known at all, further completed by reproduction tests (see  
21 results on Soil 3T). At higher levels of soil pollution, or in the presence of a mixed pollution  
22 by trace elements, a battery of bioassays, combining acute and chronic toxicity tests,  
23 should be used, results being combined or compared according to purposes. The use of  
24 ecoscores could be useful for such comparisons, because (1) they take into account an  
25 array of different toxicity parameters which could be calculated on raw data, and (2) they  
26 can be used in further statistical treatment.

27

### 1 4.3. Concluding part

2

3 As a conclusion to this section the two questions which were set at the beginning  
4 can now be answered. First, it has been shown that the avoidance test, which displayed a  
5 sensitive response to most PAH-contaminated soils, gave a better response to all soils  
6 and thus could be used as a more sensitive indicator of soil quality. Second, it has been  
7 shown that each test or group of tests exhibited a specific response and as a  
8 consequence a single test is not enough to assess soil quality. This necessitates adapting  
9 the strategy used by the laboratory to the expected level and nature of pollution, in  
10 particular when in the presence of a mixed pollution.

11

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13

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18

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4

1 **Figure captions**

2

3 **Fig. 1.** Concentrations of 2- to 6-ring PAHs in the four studied soils. Values are means of  
4 three replicate dosages, with S.D. as error bars

5

6 **Fig. 2.** Correlation between mean ecoscores (bulked for five different bioassays) and  
7 concentrations of 3-ring PAHs in the four studied soils. \* = significant at 0.05 risk  
8 level

9

1 **Table 1.** Main physicochemical characteristics of the four studied soils

2

	<b>Texture</b>	<b>Moisture (%)</b>	<b>pH<sub>water</sub></b>	<b>Total carbon (%)</b>	<b>Total organic carbon (%)</b>	<b>Total organic nitrogen (mg.kg<sup>-1</sup>)</b>	<b>Total phosphorus (mg.kg<sup>-1</sup>)</b>
<b>Soil 1</b>	silty sand	9.0±0.2	8.1±0.02	9.7±0.2	9.2	1300	770
<b>Soil 2</b>	sand	18.9±0.6	7.8±0.03	44.3±1.4	44.2	5600	1900
<b>Soil 3</b>	sand	17.4±0.1	7.9±0.02	11.2±0.6	9.0±0.5	1700	620
<b>Soil 3T</b>	sand	16.3±0.3	8.3±0.01	8.6±0.2	5.7±0.1	2088	670

3

4 Means of three replicated measures followed by standard deviations. Concentrations are  
5 expressed on a dry soil basis

6

1 **Table 2.** Concentration ( $\text{mg.kg}^{-1}$ ) of cyanides and heavy metals in the four studied soils,  
 2 compared with the geochemical background

3

	$\Sigma 16\text{PAHs}$	Cyanides	Heavy metals							
			As	Cd	Co	Cr	Cu	Ni	Pb	Zn
<b>Soil 1</b>	701±84	6.6	21.7±1.6	<4	8.0±0.1	31.9±0.7	47.8±3.8	21.1±0.3	35.1±3.8	75.1±11.9
<b>Soil 2</b>	3687±85	67	33.4±2.6	5.2±0.01	48.9±1.4	52.2±4.0	126.4±0.3	46.9±1.9	345.7±7.0	514±10.6
<b>Soil 3</b>	2895±54	0.8	6.5±0.5	<4	9.6±0.2	32.9±0.3	19.3±0.4	19.8±0.2	23.9±0.8	92.7±0.8
<b>Soil 3T</b>	345±41	0.5	6.5±0.9	<4	8.9±0.1	31.3±0.9	21±0.6	26.8±3.3	25.9±3.7	104±5.8
<b>Control 1</b>	1.2±0.1	0.2	20.3±2.8	<4	7.6±0.4	39.4±3.3	8.9±0.3	19.1±0.8	7.1±0.003	39.0±1.9
<b>Control 2</b>	8.6±0.7	0.2	18.6±0.9	<4	9.9±0.4	25.5±0.5	26.0±1.2	17.3±0.7	38.6±1.2	87.6±2.0
<b>Control 3</b>	1.0±0.7	0.1	6.7±0.8	<4	9.1±0.4	39.2±1.3	14.8±0.2	27.0±2.3	19.9±0.9	67.9±1.1
<b>Geochemical background</b>			8.9±1.2	0.4±0.03	9.3±0.9	48.8±2.7	16.7±1.8	24.7±5.7	38.4±5.6	73.7±6.2

4  
 5 Means of three replicated measures followed by standard deviations. Background  
 6 concentrations have been measured over a wide range of unpolluted agricultural and  
 7 forest soils (Sterckeman et al., 2002). Concentrations are expressed on a dry soil basis

8

1 **Table 3.** Concentrations of 16 PAHs of the US-EPA list in the four studied soils

2

	<b>Soil 1</b>	<b>Soil 2</b>	<b>Soil 3</b>	<b>Soil 3T</b>
Naphthalene	19.2±0.7	150.9±10.6	594.2±13.8	11.1±4.9
Acenaphthylene	1.9±0.04	23.5±1.1	3.1±0.1	0.2±0.4
Acenaphthene	1.9±0.04	2±0.1	217.4±1.2	7.4±0.6
Fluorene	19.3±0.3	83.1±3.7	226.8±2.8	4.5±4.2
Phenanthrene	119.4±17.7	308.2±17.7	629.3±4.2	3.8±0.8
Anthracene	183.2±8.3	206.7±7	202.5±31.7	19.1±5.9
Fluoranthene	130.8±22.5	625.2±30.7	414.3±1.2	55.1±11.9
Pyrene	54.5±9.9	299.4±10.9	233.4±0.4	50.6±9.9
Benzo[ <i>a</i> ]anthracene	48.4±9.2	391.9±13.1	85.7±0.9	21.2±2.9
Chrysene	47.7±8.2	410.4±8.1	75.4±0.9	19.0±2.7
Benzo[ <i>b</i> ]anthracene	2.6±1	210.8±5.8	56.2±0.3	42.9±5.4
Benzo[ <i>k</i> ]fluoranthene	2.8±0.8	161.9±2.6	25.8±0.3	15.7±1.7
Benzo[ <i>a</i> ]pyrene	38.2±2.6	364.1±2.9	60.4±6.7	37.1±4.8
Dibenzo[ <i>ah</i> ]anthracene	4.9±0.5	59.3±0.3	6.9±0.2	1.4±0.1
Benzo[ <i>ghi</i> ]perylene	15.1±1.5	196.1±6.5	32.5±1	21.4±4.2
Indeno[123- <i>cd</i> ]pyrene	11.2±1	193.7±1.6	30.8±0.3	34.8±5.3

3

4 Means of three replicated measures followed by standard deviations. Concentrations are

5 expressed on a dry soil basis

6

7

1 **Table 4.** Toxicity of the four studied soils according to the *Lactuca sativa* germination  
 2 bioassay

3

	<b>EC<sub>50</sub></b> (g.100 g <sup>-1</sup> )	<b>TU</b> 100/EC <sub>50</sub>	<b>EC<sub>20</sub></b> (g.100 g <sup>-1</sup> )	<b>EC<sub>10</sub></b> (g.100 g <sup>-1</sup> )	<b>NOEC</b> (g.100 g <sup>-1</sup> )	<b>Inhibition</b> (%)	<b>Ecoscore</b> (%)
<b>Soil 1</b>	>100	<1	64.9 (48.9–86.2)	25.3 (13.5–47.5)	<35	20.4	47
<b>Soil 2</b>	>100	<1	80.8 (62.9->100)	41.7 (29.9–58.1)	<35	21.4	47
<b>Soil 3</b>	21.3 (15.5–29.3)	4.5	3.5 (1.9–6.5)	1.4 (0.6–3.4)	<5	70.9	93
<b>Soil 3T</b>	NT	NT	NT	NT	NT	0	0

4

5 EC<sub>50</sub>, EC<sub>20</sub>, EC<sub>10</sub>= concentration causing 50%, 20%, 10% inhibition of germination rate,  
 6 respectively (confidence interval at 0.05 risk level between brackets); TU = 100/EC<sub>50</sub>;  
 7 NOEC = no observed effect concentration; NT = not toxic, observed response statistically  
 8 indistinguishable from unpolluted soil sampled on the studied site; inhibition = relative  
 9 decrease in response (%) compared to control soil at the highest dose tested

10

1 **Table 5.** Toxicity of the four studied soils according to the *Lactuca sativa* growth inhibition  
 2 bioassay

3

	<b>EC<sub>50</sub></b> (g.100 g <sup>-1</sup> )	<b>TU</b> 100/EC <sub>50</sub>	<b>EC<sub>20</sub></b> (g.100 g <sup>-1</sup> )	<b>EC<sub>10</sub></b> (g.100 g <sup>-1</sup> )	<b>NOEC</b> (g.100 g <sup>-1</sup> )	<b>Inhibition</b> (%)	<b>Ecoscore</b> (%)
<b>Soil 1</b>	>100	<1	>100	87.4 (72.5->100)	100	0.7	13
<b>Soil 2</b>	>100	< 1	>100	95.1 (83.5->100)	60	10	20
<b>Soil 3</b>	16.6 (13.1–21.0)	5.8	2.9 (1.7–4.8)	1.2 (0.6–2.4)	<5	79.3	93
<b>Soil 3T</b>	NT	NT	NT	NT	NT	0	0

4  
 5 EC<sub>50</sub>, EC<sub>20</sub>, EC<sub>10</sub>= concentration causing 50%, 20%, 10% inhibition of growth rate,  
 6 respectively (confidence interval at 0.05 risk level between brackets); TU = 100/EC<sub>50</sub>;  
 7 NOEC = no observed effect concentration; NT = not toxic, observed response statistically  
 8 indistinguishable from unpolluted soil sampled on the studied site; inhibition = relative  
 9 decrease in response (%) compared to control soil at the highest dose tested

10

1 **Table 6.** Toxicity of the four studied soils according to the *Eisenia fetida* mortality bioassay

2

	<b>LC<sub>50</sub></b> (g.100 g <sup>-1</sup> )	<b>TU</b> 100/LC <sub>50</sub>	<b>LC<sub>20</sub></b> (g.100 g <sup>-1</sup> )	<b>LC<sub>10</sub></b> (g.100 g <sup>-1</sup> )	<b>NOEC</b> (g.100 g <sup>-1</sup> )	<b>Inhibition</b> (%)	<b>Ecoscore</b> (%)
<b>Soil 1</b>	NT	NT	NT	NT	NT	0	0
<b>Soil 2</b>	>100 (97.8->100)	<1	88.0 (83.0–93.2)	79.1 (72.2–86.7)	60	37.5	33
<b>Soil 3</b>	0.60 (0.56–0.64)	167	0.51 (0.46–0.55)	0.46 (0.41–0.51)	0.4	100	100
<b>Soil 3T</b>	NT	NT	NT	NT	NT	0	0

3

4 LC<sub>50</sub>, LC<sub>20</sub>, LC<sub>10</sub>= concentration causing 50%, 20%, 10% inhibition of survival rate,  
5 respectively (confidence interval at 0.05 risk level between brackets); TU = 100/LC<sub>50</sub>;

6 NOEC = no observed effect concentration; NT = not toxic, observed response statistically  
7 indistinguishable from unpolluted soil sampled on the studied site; inhibition = relative  
8 decrease in response (%) compared to control soil at the highest dose tested

9

1 **Table 7.** Toxicity of the four studied soils according to the *Folsomia candida* population  
 2 growth bioassay

3

	<b>EC<sub>50</sub></b> (g.100 g <sup>-1</sup> )	<b>TU</b> 100/EC <sub>50</sub>	<b>EC<sub>20</sub></b> (g.100 g <sup>-1</sup> )	<b>EC<sub>10</sub></b> (g.100 g <sup>-1</sup> )	<b>NOEC</b> (g.100 g <sup>-1</sup> )	<b>Inhibition</b> (%)	<b>Ecoscore</b> (%)
<b>Soil 1</b>	93.5 (75.3->100)	1.1	37.0 (30.0-45.7)	22.8 (17.2-30.3)	10	63.6	60
<b>Soil 2</b>	NT	NT	NT	NT	NT	0	0
<b>Soil 3</b>	2.2 N/A	45.5	2.1 N/A	1.9 N/A	1	100	100
<b>Soil 3T</b>	>100	<1	>100	5.2 (1.8-15.1)	<0.35	27	53

4

5 EC<sub>50</sub>, EC<sub>20</sub>, EC<sub>10</sub>= concentration causing 50%, 20%, 10% inhibition of population growth  
 6 rate, respectively (confidence interval at 0.05 risk level between brackets); TU = 100/EC<sub>50</sub>;  
 7 NOEC = no observed effect concentration; NT = not toxic, observed response statistically  
 8 indistinguishable from unpolluted soil sampled on the studied site; inhibition = relative  
 9 decrease in response (%) compared to control soil at the highest dose tested; N/A =  
 10 cannot be calculated

11

1 **Table 8.** Avoidance of the four studied soils by *Folsomia candida*

2

	<b>EC<sub>50</sub></b> (g.100 g <sup>-1</sup> )	<b>TU</b> 100/EC <sub>50</sub>	<b>EC<sub>20</sub></b> (g.100 g <sup>-1</sup> )	<b>EC<sub>10</sub></b> (g.100 g <sup>-1</sup> )	<b>NOEC</b> (g.100 g <sup>-1</sup> )	<b>Inhibition</b> (%)	<b>Ecoscore</b> (%)
<b>Soil 1</b>	27.1 (19.5–37.6)	3.7	2.9 (2.0–4.3)	0.9 (0.5–1.6)	<0.35	9.5	80
<b>Soil 2</b>	>100	<1	1.7 (0.6–5.1)	0.04 (0.004–0.403)	<0.35	12.5	67
<b>Soil 3</b>	0.8 (0.6–1)	129	0.3 (0.2–0.4)	0.042 (0.008–0.205)	<0.35	100	100
<b>Soil 3T</b>	NA	NA	NA	NA	NA	0	0

3

4 EC<sub>50</sub>, EC<sub>20</sub>, EC<sub>10</sub>= concentration causing 50%, 20%, 10% avoidance rate, respectively5 (confidence interval at 0.05 risk level between brackets); TU = 100/EC<sub>50</sub>; NOEC = no

6 observed effect concentration; NA = not avoided, observed response statistically

7 indistinguishable from unpolluted soil sampled on the studied site; inhibition = relative

8 decrease in response (%) compared to control soil at the highest dose tested

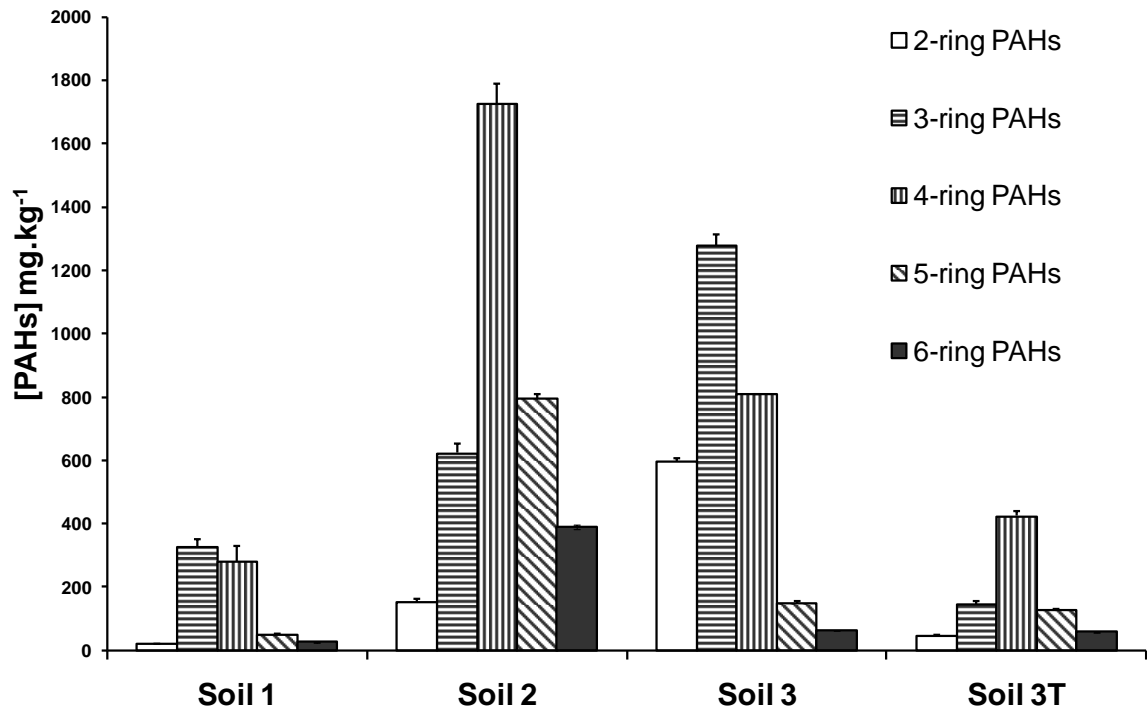
9

1 **Table 9.** Eco-scores of the four studied soils obtained with five different bioassays. Total  
 2 ecoscores are average values of the five tested bioassays (rows) or of the four soils  
 3 (columns)

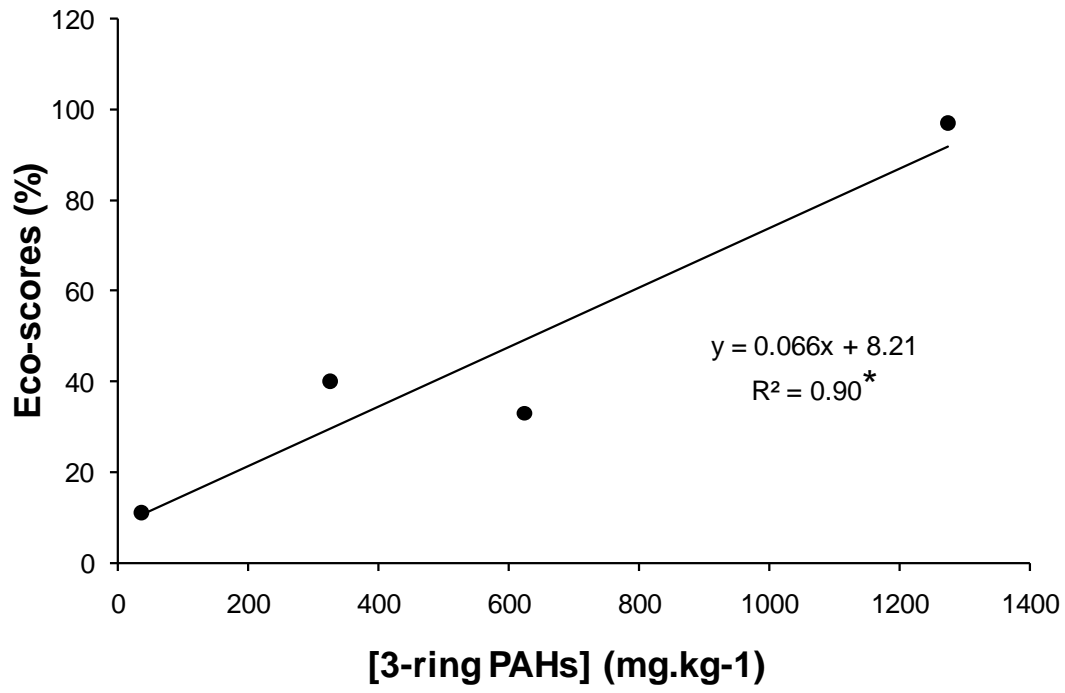
	<b>Lettuce germination</b>	<b>Lettuce growth inhibition</b>	<b>Earthworm mortality</b>	<b>Springtail reproduction</b>	<b>Springtail avoidance</b>	<b>Total</b>
<b>Soil 1</b>	47	13	0	60	80	40
<b>Soil 2</b>	47	20	33	0	67	33
<b>Soil 3</b>	93	93	100	100	100	97
<b>Soil 3T</b>	0	0	0	53	0	11
<b>Total</b>	47	32	33	53	62	

5

6



1  
2 Fig. 1  
3



1  
2 Fig. 2