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► **To cite this version:**

Maite Martínez Aldaya, Christine Lors, Sandrine Salmon, Jean-François Ponge. Avoidance bio-assays may help to test the ecological significance of soil pollution. *Environmental Pollution*, 2006, 140 (1), pp.173-180. 10.1016/j.envpol.2005.06.011 . hal-00363745

HAL Id: hal-00363745

<https://hal.science/hal-00363745v1>

Submitted on 25 Feb 2009

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1 **Avoidance bio-assays may help to test the ecological significance of soil**
2 **pollution**

3

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5

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15

16 **Capsule**

17

18 Polluted soils are avoided by soil animals, a phenomenon which can be used as a
19 cheap, sensitive tool for the early detection of environmental risk

20

21 **Abstract**

22

23 We measured the short-term (100') avoidance of a soil heavily polluted by
24 hydrocarbons by the soil springtail *Folsomia candida*, at six rates of dilution in a control,
25 unpolluted soil. We compared the results with those of long-term (40-day) population tests.
26 Five strains were compared, of varying geographical and ecological origin. When pure, the
27 polluted soil was lethal in the long-term and avoided in the short-term by all strains.

1 Avoidance tests, but not population tests, were able to discriminate between strains.
2 Avoidance thresholds differed among strains. Two ecological consequences of the results
3 were discussed, i) toxic compounds may kill soil animals or deprive them from food, resulting
4 in death of populations, ii) pollution spots can be locally deprived of fauna because of escape
5 movements of soil animals. Advantages and limitations of the method have been listed,
6 together with proposals for their wider use in soil ecology and ecotoxicology.

7

8 *Key-words:* Avoidance; Ecotoxicological test; *Folsomia candida*; Soil pollution; Toxicity

9

10 **1. Introduction**

11

12 The repellence of toxic substances to soil animals has been known for a long time
13 and was quantitatively assessed on several animal groups (Eijsackers, 1978; Hund-Rinke et
14 al., 2003). The ecological assessment of soil toxicity is currently achieved through population
15 and bioaccumulation bio-assays (Crouau et al., 2002). In standardized ecotoxicological tests
16 using the earthworm *Eisenia fetida* (ISO 11268) and the springtail *Folsomia candida* (ISO
17 11267), populations of these soil animals are submitted to increasing doses of a toxicant,
18 which they cannot escape during the duration of the bio-assay (one to three months). In
19 nature, contrary to plants, most animals move to best places for feeding, reproduction and
20 ecdysis, using chemical cues (Bengtsson et al., 1991; Salmon and Ponge, 2001). They are
21 also able to escape deleterious environment or food using the same cues (Fábian and
22 Petersen, 1994), except when they are immediately killed or immobilized by high doses of a
23 toxicant (Best et al., 1978). Both attraction and repulsion are behavioural traits which
24 increase the ecological fitness of sensitive species (Tranvik and Eijsackers, 1989). It has
25 been demonstrated that most fungal strains and decomposition stages of leaf litter which
26 were either attractive or repellent to soil springtails in the short-term were those which in the
27 long-term favoured or disfavoured their growth, survival and reproduction, respectively
28 (Sadaka-Laulan and Ponge, 2000).

1
2 From a functional point of view, a sound basis for the early risk assessment of soil
3 pollution at the ecosystem level should be to test whether a given soil will allow or not a
4 community of soil organisms to colonize it (Filser and Hölscher, 1997), which lies on both
5 dispersal to a habitat and population growth (Marinissen and Van den Bosch, 1992). It has
6 been observed repeatedly that plant litter decomposition and building of a soil structure can
7 be lost in polluted soils by the absence of key functional groups (Gillet and Ponge, 2002).
8 Such functional losses may result not only from direct or indirect toxicity of a soil but also
9 from the repellence of the same soil towards potential colonizers (Gillet and Ponge, 2004).
10 Recently, several papers pointed on the need to combine ecotoxicological tests with
11 avoidance tests for a proper assessment of soil toxicity (Heupel, 2002; Greenslade and
12 Vaughan, 2003; da Luz et al., 2004).

13
14 The present study was aimed at comparing short-term avoidance tests with long-term
15 tests of toxicity, using a soil polluted by polycyclic aromatic hydrocarbons (PAHs). *Folsomia*
16 *candida* (Willem), a soil-dwelling springtail (Collembola: Isotomidae) currently used for the
17 laboratory assessment of soil toxicity (Smit and Van Gestel, 1998), was considered
18 representative of an invertebrate animal group which is widely distributed in the soil
19 ecosystem and which plays a significant role in the regulation of microbial processes
20 (Petersen and Luxton, 1982). Different strains of *F. candida* were compared, in order (i) to
21 check for strain variation, given the high degree of polymorphism which is known to occur in
22 this cosmopolitan species (Goto and Ögel, 1961), (ii) to evaluate the sensitivity and validity of
23 avoidance tests when performed on different clones of the same species (Chenon et al.,
24 2000).

25
26 **2. Material and methods**

27

1 The test soil used in our experiments was obtained from a former coke oven site in
2 northern France (Nord-Pas-de-Calais) in September 2003, then kept frozen at the laboratory.
3 A control soil was collected in a nearby non-polluted, plant-restorated zone, within the same
4 industrial site. Most physico-chemical properties of the test and the control soil are shown in
5 Tables 1 and 2. The moisture content was measured by gravimetry and the texture was
6 determined by laser particle size analysis. Soil pH_{water} was measured using a Consort® C83
7 pH-meter fitted with glass electrodes corrected for temperature and a Schott® box with
8 Ingold® combined electrodes. Total organic carbon was deduced from total carbon and
9 inorganic carbon values, which were determined with a TOC-5000A Shimadzu® analyser.
10 Total organic nitrogen was determined by the Kjeldahl method, and total phosphorus as well
11 as metal concentrations (As, Cd, Co, Cr, Cu, Ni, Pb and Zn) were analysed by Inductive
12 Coupled Plasma Atomic Emission Spectrometry (ICP-AES) in a 138 Ultrace Jobin Yvon®
13 analyser after hot hydrofluoric and perchloric acid digestion of the solid phase.
14 Concentrations of the 16 PAHs of the US EPA list of compounds (Greene et al., 1989) were
15 measured using High Performance Liquid Chromatography in a 2690 HPLC Waters®
16 analyser fitted with an ultraviolet inverted phase C 18 Supelco® column (length 250 mm,
17 internal diameter 2.1 mm), coupled to a 996 Waters® UV photodiode array detector, after
18 extraction by dichloromethane/acetone (50/50 v/v) using the Accelerated Solvent Extractor
19 Dionex® ASE 200. Chemical analyses were made in triplicate.

20
21 The test soil was used pure and mixed with the control soil at 50, 10, 5, 1 and 0.35%
22 concentration (w/w), just before each experiment run, after thawing overnight at 3°C.
23 Deionized water was added in sufficient amount to obtain a paste for avoidance bio-assays
24 or a solid substrate moistened at field capacity for population bio-assays. Rearing
25 microcosms were checked weekly and deionized water was added to avoid desiccation. The
26 use of a paste, instead of the natural soil, in avoidance experiments, was justified by the
27 need to prevent animals from hiding in the soil when placed under constant illumination, and
28 to achieve standard textural conditions of the substrate.

1
2 Five *F. candida* strains collected in France were compared. In all cases, the clones
3 came from a single thelytokous (parthenogenetic) female which was collected from a soil
4 sample after extraction of microarthropods in a Berlese funnel, then sorted live. The animals
5 were reared on fine quartz sand moistened with tap water and they were fed *ad libitum* with
6 finely ground cow dung which was free of pesticides and antibiotics. Strain 1 was collected in
7 October 2002 in a neutral soil slightly polluted by hydrocarbons (soil over tar deposit in an
8 abandoned oil refinery) at Pechelbronn (48°56'N, 7°50'E, pH_{water} 6.7, Σ 16 PAHs 6.0 mg.kg⁻¹).
9 Strain 2 was collected at the same date in a neutral, unpolluted control soil, 15 m from the
10 previous site (48°56'N, 7°50'E, pH_{water} 7.0, Σ 16 PAHs 0.9 mg.kg⁻¹). Strain 3 was collected in
11 May 2002 in an unpolluted neutral soil in the park of the laboratory (oak/hornbeam woodlot)
12 at Brunoy (48°40'N, 2°30'E, pH_{water} 7.7, Σ 16 PAHs 1.2 mg.kg⁻¹). Strain 4 was collected in
13 October 2002 in an unpolluted acid soil at Pfaffenbronn (oak/beechn woodland), five
14 kilometres from the Pechelbronn site (48°59'N, 7°50'E, pH_{water} 4.3, PAHs not determined).
15 Strain 5 was collected in May 2002 in an acid soil (oak/pine woodland) at Brunoy (48°40'N,
16 2°30'E, pH_{water} 4.3, PAHs not determined). Previous to each experimental run all specimens
17 used had never been into contact with the polluted soil nor with any other kind of pollutant.
18 Although the strains originated from very different sites, some of them were polluted, rearing
19 conditions were standardized (quartz sand, moistened with tap water, with dry powdered cow
20 dung added *ad libitum*), and the time between first inoculation by a female and experimental
21 use of its offspring was such (at least a year) that we discarded any possible residual
22 pollution in the rearing boxes. The test specimens were selected among fully developed
23 (adult) animals of the same rearing box.

24
25 Avoidance experiments were performed in sterile polystyrene Petri dishes (55 mm
26 diameter, 10 mm height), the bottom of which was lined with two half-disks of filter paper
27 made of glass fiber (50 mm diameter). The entire surface of each disk was then covered with
28 a soil paste. One control half disk was covered with the control soil, the other with the test

1 soil or a mixture of both soils. The two half-disks were separated by a 2 mm space line, at
2 the center of which one individual of *F. candida* was deposited. The position of the animal
3 was recorded each 20 min up to 100 min. Previous assays showed that this duration was
4 enough although necessary to let the animal chose definitely between both sides after
5 preliminary exploration of the Petri dish. Twenty replicates, in two successive batches of ten,
6 were followed together. During the experiment, Petri dishes were placed under a Sharp®
7 fluorescent illuminator in a chamber at 20°C. Blank experiments using control soil at both
8 sides checked for the absence of any light gradient effect which could bias the results
9 (Salmon and Ponge, 1998). Totals of five counts over 100 min for each Petri dish were used
10 for testing differences between control and polluted sides, using sign tests (Sokal and Rohlf,
11 1995). Notice that unit data, ranging from 0 to 5, measured the average position of a single
12 individual (from nil to constant avoidance) during the duration of the experiment and thus
13 were not replicated over time. Replication and corresponding calculation of the degrees of
14 freedom concerned only the 20 petri dishes which were independently followed in the course
15 of time. Previous experiments with the same design showed that average values for choice
16 position should be preferred to end-point values, given that choice by Collembola is rapid,
17 often occurring within 10 min, but erratic movements may still occur thereafter (Salmon and
18 Ponge, 1998, 2001; Sadaka-Laulan and Ponge, 2000).

19

20 In the course of each avoidance experimental run, some animals were found dead or
21 paralyzed, their number increasing with the concentration of the polluted soil in the dilution
22 series. These animals were counted separately, then discarded from the data set used for
23 the sign test.

24

25 Population response was assessed by introducing batches of ten adults into each of
26 five rearing chambers (polystyrene boxes 45 mm diameter, 25 mm height), fifth-filled with the
27 control soil or with the same mixtures of control/polluted soil as used for avoidance
28 experiments. A small amount of dry cattle dung powder was added above the soil substrate,

1 then boxes were incubated at 20°C in darkness during 40 days. At the end of the experiment,
2 the whole population (including the ten females introduced at the start of the experiment) was
3 collected. We used forceps for collecting animals which were visible over the substrate.
4 Animals living deep in the substrate were collected by flotation under excess water, and all
5 specimens were immediately counted. Population sizes were compared between control and
6 test soils using t tests (Sokal and Rohlf, 1995).

7

8 **3. Results**

9

10 The studied substrate exhibited a high content in PAH compounds but was not
11 polluted by heavy metals, arsenic or cyanides (Tables 1 and 2).

12

13 Figure 1 shows the results of all avoidance tests which were performed on the five
14 strains of *F. candida*. There was no bias due to a possible light gradient effect, showing that
15 light was homogeneously distributed throughout the observation area. All blank tests, using
16 the control soil on both sides of Petri dishes, did not reveal any significant departure from
17 random values, nor any mortality during the test period. At a test soil concentration of 10% or
18 higher, specimens from all strains avoided the test side. Strain 4 (acid soil Pfaffenbronn) did
19 not avoid the test side to the same extent than the other four strains, even at the highest
20 concentration (100%), while avoidance was total for the other four strains. At 1% and 5% test
21 soil, Strain 4 did not avoid the test side, while the other four strains did. At 0.35% test soil,
22 avoidance was displayed by Strain 2 only. Thus the five strains could be classified in a
23 decreasing order of sensitivity to the test soil: 2 > 1-3-5 > 4. For all strains, the decrease from
24 the point of minimum (or nil) avoidance to the point of maximum (or total) avoidance was
25 progressive.

26

27 The number of animals which were observed to die or become motionless during
28 avoidance runs increased according to the concentration of the test soil (Fig. 2). All animals

1 were alive and actively moving when the test soil was diluted more than 20 times (< 5%).
2 However, Strain 4 began to be affected only at 10% test soil, contrary to the four other
3 strains, pointing again to a lower sensitivity of this strain.

4
5 Figure 3 shows the results of population tests. At 10, 50 and 100% test soil, all
6 introduced animals were killed, whatever the strain. At 5% test soil, some animals were still
7 alive in some boxes at the end of the experimental period (40 days), but reproduction was
8 rarely observed. Below 5% test soil, reproduction occurred in all boxes, without any
9 significant departure from the control soil. In the control soil the number of juveniles produced
10 after 40 days was 119 ± 30 , 109 ± 17 , 39 ± 5 , 116 ± 30 and 90 ± 13 (mean \pm S.E.) with Strains 1 to
11 5, respectively. The reproduction rate and the coefficient of variation met the validity criterion
12 of the ISO guideline (ISO 11267) except for Strain 3, which displayed a lower reproduction
13 rate than all other strains (one-way ANOVA on log-transformed data followed by SNK
14 procedure, $P = 2 \cdot 10^{-4}$). Contrary to avoidance tests, threshold concentrations did not differ
15 between strains and the passage from the absence of effect to total or near total effect was
16 abrupt whatever the strain.

17

18 **4. Discussion**

19

20 Toxicity of the test soil to the Collembolan *Folsomia candida* was clearly shown by
21 population tests (Fig. 3). When the test soil was diluted to 5% in the control soil the batch of
22 10 adult animals did not reproduce (strains 1 and 3) or even partially or totally died (other
23 strains). At higher concentration, mortality at 40 days was total. The PAH content of the test
24 soil could explain this toxicity, however, we cannot discard the effect of other compounds, not
25 dosed in the present study, such as tar. A visual inspection of the polluted soil under a
26 dissecting microscope revealed an abundance of dark more or less spherical pellets ≤ 1 mm
27 which, when broken with scissors, revealed a black, vitrified core part surrounded by a
28 mixture of organic matter and clay particles. Such strongly heterogeneous material may

1 explain the absence of a linear, or even curvilinear dose-response relationship when the
2 animals were let several weeks into contact with the test substrate. We hypothesize that
3 there was a threshold of dilution above which the animals were able to avoid a few,
4 dispersed toxic micro-sites and thus behaved like in an innocuous environment (Tranvik and
5 Eijsackers, 1989). Such micro-heterogeneity of the substrate did not occur in our avoidance
6 tests, probably because of the more intense mixing which resulted from the preparation of
7 the muddy paste.

8
9 Despite the fact that the test soil was clearly toxic to *F. candida* and the control soil
10 was not, we cannot discard possible effects of soil type to explain why the animals preferred
11 the control soil in avoidance experiments. The use of another, not polluted soil, coming from
12 the same industrial site, was preferred to a neutral substrate such as quartz sand. To be
13 realistic an avoidance experiment should mimic what the animals have to their disposal in the
14 site from which the test soil was originating, i.e. a patchwork of polluted and unpolluted
15 micro-sites, reflected in our test and control soils, respectively.

16
17 Our results point at a good correlation between avoidance and toxicity tests.
18 However, only avoidance tests discriminated between strains of *F. candida*. Genetic
19 polymorphism has been demonstrated between clones of *F. candida* coming from several
20 European laboratories but this could not be correlated with ecotoxicological responses to
21 cadmium and phenanthrene (Chenon et al., 2000). In particular these authors showed that
22 the lowest concentration affecting mortality and reproduction did not differ between strains,
23 as in our study, but they did not perform avoidance experiments.

24
25 What is the ecological significance of pollution avoidance? In a study comparing two
26 isotomid species coming from the Gusum area, a metal-contaminated site in Sweden,
27 Tranvik and Eijsackers (1989) observed that *Folsomia fimatarioides* was able to avoid diets
28 and substrates highly polluted by heavy metals (Cu+Zn), while *Isotomiella minor* was not.

1 The former species was more abundant than the latter when exposed to the highest
2 concentration of metals in the soil. The authors concluded that avoidance of polluted diets
3 and substrates offered an advantage to *F. fimetarioides* over *I. minor* in polluted soils,
4 because it allowed the former species to select less polluted micro-sites and food in a
5 heterogeneous environment. This indicates that even tiny animals, such as springtails, are
6 capable of perceiving differences in their habitat. At the scale of life of micro-arthropods, the
7 landscape is made of islands of food resources and habitats between which the animals
8 move and which they select for nutrition, moulting, and oviposition (Joosse, 1971). Shape
9 and colour are of no use within the soil where animals live in darkness, therefore they must
10 use chemical and mechanical sense organs for detecting deleterious and attractive habitat
11 and food. Species-specific aggregation in conditioned sites increases the probability to find
12 suitable places and favours mating (Joosse, 1971). Avoidance and attraction may be
13 directional and selective (Verhoef and Nagelkerke, 1977). Repellent substances may also act
14 by discouraging food intake (Fountain and Hopkin, 2001) or by favouring non-directional
15 dispersal (Sjögren, 1997), thus increasing the chance an animal will find a suitable place
16 where it will remain motionless (Michelozzi et al., 1997). However, a high dose of pollutant or
17 a longer time of exposure may decrease mobility (Petersen and Gjelstrup, 1998). Some
18 animals became motionless in the course of our short-term avoidance test (Fig. 2). Thus,
19 when the toxicant exhibits paralyzing effects, escape movements may prevent the animals
20 from intoxication but only at some distance from the poison source. This is a key point for
21 assessing the ecological significance of avoidance, in particular in the case of PAH mixtures,
22 which act as narcotics and thus may inhibit locomotory activity (Landrum et al., 2003).

23

24 Pollution may affect soil animal communities and their functioning in two ways. First,
25 toxicity kills animals or prevents them from feeding and reproduction. This only occurs at the
26 right place where the pollutant has been deposited. Indirect effects through collapses in
27 microbial and plant communities (Gillet and Ponge, 2003) or predator populations
28 (Abrahamsen et al., 1980) are also spatially limited and fall in this category. Second,

1 repellence of toxicants may help the animals to find refuges deeper in the soil or outside the
2 pollution spot. In this case, the final result will depend on whether the animal is adapted to
3 live in deeper soil or is able to move rapidly at the soil surface; thus it will depend on species-
4 specific biological traits. Such spatial shifts have been described or they can be suspected
5 from existing data. Gillet and Ponge (2003) observed that several surface-dwelling species of
6 Collembola, collected in an organic soil strongly polluted by heavy metals (Zn+Cd), visited
7 the underlying clay-rich mineral soil, and fed on the mineral substrate rather than on the
8 metal-contaminated organic layer. Vertical migration of Collembola down to the mineral soil
9 was experimentally demonstrated by Best et al. (1978) following naphthalene application on
10 the ground surface, but this phenomenon occurred only at the lowest rate of application. A
11 decrease in horizontal migration was demonstrated at all rates of application. Thus the few
12 existing data point to vertical migration of microarthropods to deeper soil layers rather than to
13 outward horizontal migration. Consequences are that low rates of application of a pollutant,
14 eventually not reaching toxic doses, may impoverish the topsoil in some animal species. This
15 may result into functional shifts such as organic matter accumulation and proliferation of
16 mycelial webs (Gillet and Ponge, 2002).

17
18 Whether avoidance tests should be preferred to mortality/reproduction tests for the risk
19 assessment of soil pollution is prone to discussion. The laboratory assessment of repellence
20 is cheaper (total duration of a test three to four hours, preparation and calculation included)
21 and needs a much lesser amount of specimens than standardized ecotoxicological tests
22 based on population response. Tests of a longer duration are even disqualified in the case of
23 neurotoxic compounds, as mentioned above. From an ecological point of view, avoidance
24 tests seem more relevant than classical ecotoxicological tests, and they are more sensitive to
25 within-species populational differences. However, there are several limitations to a wider use
26 of avoidance tests as an alternative to the direct assessment of toxicity. Some substances,
27 such as cadmium salts, are not perceived as repellent, while they are toxic when the animals
28 are forced to keep into contact with them (Greenslade and Vaughan, 2003). This means that

1 this toxicant cannot be avoided in the environment and, thus, will destroy entire populations,
2 contrary to others which the animals currently escape. Sadaka et al. (1998) showed that a
3 basidiomycetous fungal strain was attractive to the collembolan *Onychiurus sinensis* in short-
4 term experiments while it affected negatively its growth, survival and reproduction in the long-
5 term. However, examples of compounds or substrates which are both repellent and good for
6 population growth are unknown.

7

8 The above examples show that a proper combination of avoidance and toxicity tests
9 could be the best promising tool for the prediction of environmental effects of pollution. If
10 avoidance occurs at the same or at a higher rate of application than toxicity, this means that
11 pollution will definitely kill the populations of the test organism. If avoidance occurs at a
12 lower rate of application than toxicity, as in our experiment, or if not any toxicity is detected
13 while the pollutant or the substrate proves to be repellent, this means that pollution will
14 deprive the site from the test organism even in the absence of toxic effects. Thus, avoidance
15 can be used as a tool to detect environmental hazards other than toxicity.

16

17 Clearly, the proposed avoidance test, applying a toxic soil as an artificial paste at the
18 bottom of a battery of Petri dishes is a compromise between the need for a rapid and easy
19 assessment of soil toxicity, and the need to find more realistic and ecologically-relevant
20 methods. Microcosms, filled with a natural test soil, allow avoidance to be studied at the
21 community level (Chauvat and Ponge, 2002; Ponge et al., 2002; Gillet and Ponge, 2004;
22 Garnier and Ponge, 2004), and they can be at first sight considered more realistic. However,
23 it needs several weeks for the animals to select between test and control substrates in a
24 confined environment which, in the case of neurotoxic, volatile compounds acting at
25 distance, may impeded any assessment of behavioural effects. In this case, we consider more
26 realistic to test the immediate reaction of the animals in a semi-natural system purposely
27 designed for that.

28

1 Acknowledgements

2

3 The authors are indebted to the Agence de l'Environnement et la Maîtrise de
4 l'Énergie (ADEME) for financial support and to the Agence Française de Normalisation
5 (AFNOR) for fruitful discussions about the ecological significance of avoidance tests.

6

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Table 1. Physical and chemical characteristics of the test soil and the corresponding control soil. Means of three replicated measures followed by standard deviations. Concentrations are expressed on a dry soil basis

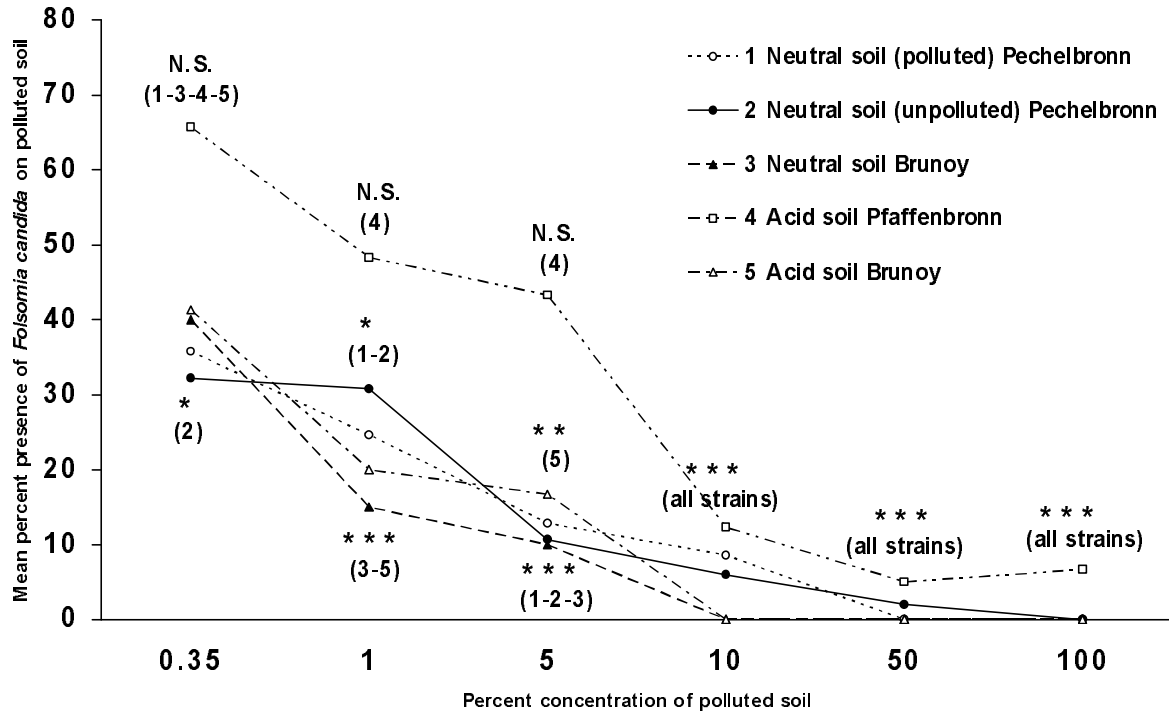
	Texture	pH _{water}	Total organic carbon (%)	Total organic nitrogen (mg.kg ⁻¹)	Total phosphorus (mg.kg ⁻¹)	Σ 16 PAHs (mg.kg ⁻¹)	Cyanides (mg.kg ⁻¹)
Test soil	Silty sand	7.9±0.02	9	1700	620	2894±54	0.8
Control soil	Medium silt	8.4±0.04	0.7	670	410	0.97±0.09	<0.1

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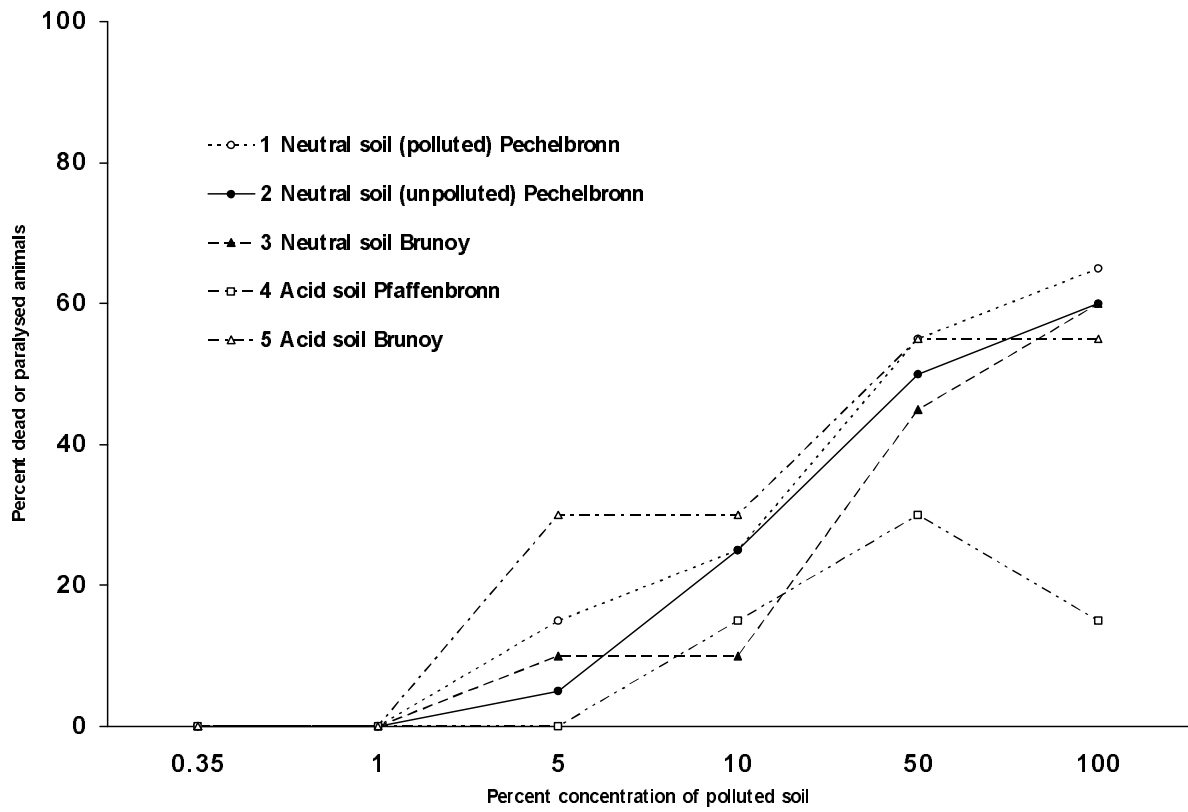
Table 2. Concentration of heavy metals (mg.kg^{-1}) in the test soil compared with geochemical background, i.e. concentrations measured over a wide range of unpolluted agricultural and forest soils (Sterkeman *et al.* 2002). Means of three replicate measures were followed by standard deviations. Concentrations are expressed on a dry soil basis

	As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Test soil	6.5±0.5	<0.4	9.6±0.2	32.9±0.3	19.3±0.4	19.8±0.2	23.9±0.8	92.7±0.8
Geochemical background	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

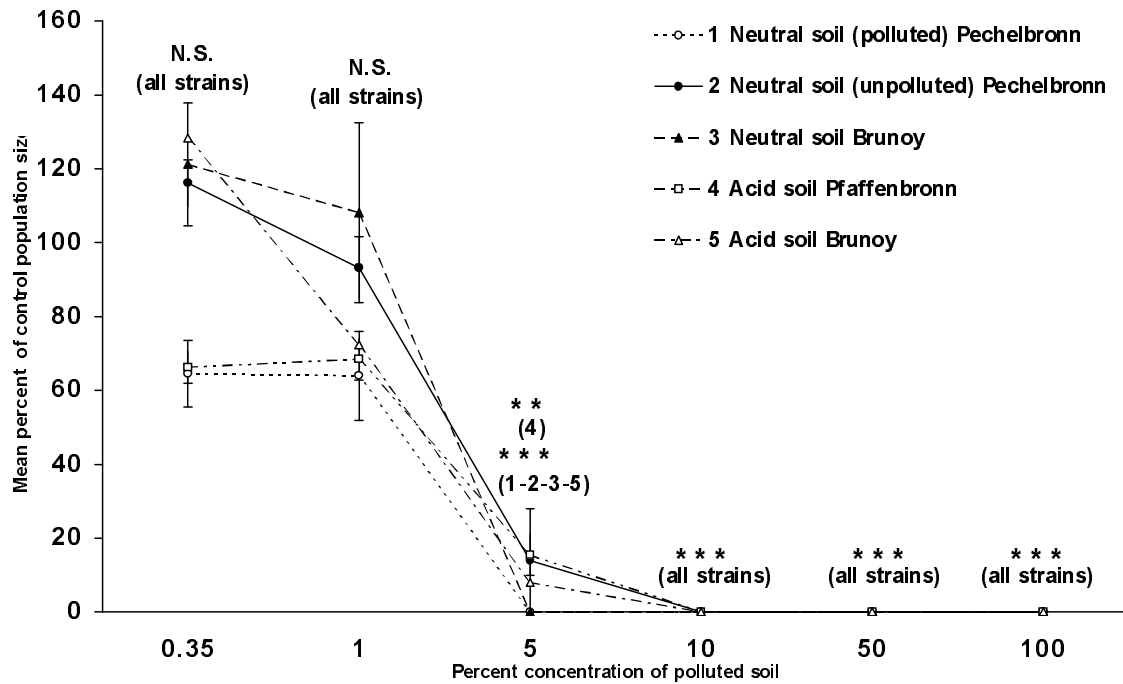
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 2 Fig. 1. Avoidance tests. Mean percent presence of five strains of *Folsomia candida* on
 3 polluted side of Petri dishes (5 counts at 20 min intervals, 20 replicates) at 6 dilution
 4 rates of the polluted soil. Differences between control and polluted side tested by sign
 5 test (N.S. = not significant; *, **, *** = significant at 0.05, 0.01 and 0.001 level,
 6 respectively). Control value is 50%



1
2 Fig. 2. Avoidance tests. Percent dead or paralysed specimens of five strains of *Folsomia*
3 *candida* during the experimental run (100 min.). Control value is 0



1
 2 **Fig. 3.** Population tests. Population size (mean of 5 replicates \pm standard error) of five strains
 3 of *Folsomia candida* after 40 days in contact with 6 dilution rates of the polluted soil,
 4 expressed as percent of the control value (all counts were divided by the mean
 5 number of animals in the 5 control microcosms). Ten adult individuals per microcosm.
 6 Differences with the control were tested by t-test (N.S. = not significant at 0.05 level,
 7 ** = significant at 0.01 level, *** = significant at 0.001 level)