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1 **Use of an avoidance test for the assessment of microbial degradation of PAHs**

2

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1 **Abstract**

2

3 An avoidance test using the soil springtail *Folsomia candida* was used to assess
4 changes in contamination levels at low doses of PAHs following incubation with
5 indigenous microflora. A soil from a former coke site was diluted to 1% in an unpolluted
6 soil from the same site, which was used as a control, then both substrates were
7 remoistened to 80% field capacity. The diluted soil was previously shown to be strongly
8 repellent to *F. candida*, although not toxic. After two-month incubation at 20°C, the mixture
9 lost its repellence capacity and became attractive to the test animal, while the global
10 PAHs content had decreased to a great extent (50%). PAH disappearance was linked to
11 the occurrence of indigenous microbiota able to degrade hydrocarbons.

12

13 *Keywords:* Avoidance; Microbial degradation; *Folsomia candida*; Soil pollution

14

15 **1. Introduction**

16

17 The assessment of soil pollution was fostered by ecotoxicological testing during
18 the last two decades (Cortet et al., 1999). In addition to toxicity tests, it has been shown
19 that the avoidance of pollutants by soil animals could provide a cheap method for the early
20 testing of environmental hazards (Yearley et al., 1996; da Luz et al., 2004; Martínez
21 Aldaya et al., 2005). Limits of the method are the need for the pollutant to be (i) perceived
22 in the environment, (ii) avoided by animals, (iii) not too noxious to their nervous system
23 (Eijsackers 1978; Tranvik and Eijsackers, 1989). When these three conditions are
24 satisfied, short-term avoidance tests could be predictive of acute and chronic toxicity
25 (Heupel, 2002; da Luz et al., 2004; Martínez Aldaya et al., 2005).

26

1 We applied an avoidance test using the soil-dwelling springtail *Folsomia candida*
2 (Willem), a standard microarthropod (ISO, 1999; Fountain and Hopkin, 2005), to the
3 microbial remediation of a soil polluted by hydrocarbons. The test was performed before
4 and after two-month incubation with indigenous microflora.

5
6 We hypothesized that any decrease in PAH content of the test soil was mirrored in
7 its repellence to *F. candida*.

8 9 **2. Materials and methods**

10 11 *2.1. Substrate used for the experiment and chemical analyses*

12
13 The polluted soil used in our experiment was collected from a former coke oven
14 site in northern France (Nord-Pas-de-Calais) in September 2003, then kept frozen at the
15 laboratory. A control soil was collected in a nearby non-polluted, plant-restored zone.
16 Soil pH_{water} was measured using a Consort® C83 pH-meter fitted with glass electrodes
17 corrected for temperature and a Schott® box with Ingold® combined electrodes. Total
18 organic carbon concentration was obtained from total carbon and inorganic carbon
19 contents, which were determined with a TOC-5000A Shimadzu® analyser. Total organic
20 nitrogen concentration was determined by the Kjeldahl method, and total phosphorus as
21 well as metal (As, Cd, Co, Cr, Cu, Ni, Pb and Zn) concentrations were analysed by
22 Inductive Coupled Plasma Atomic Emission Spectrometry (ICP-AES) in a 138 Ultrace
23 Jobin Yvon® analyser after hot hydrofluoric and perchloric acid digestion of the solid
24 phase. Concentrations of the 16 PAH of the US EPA list compounds (Greene et al., 1989)
25 were measured using High Performance Liquid Chromatography in a 2690 HPLC
26 Waters® analyser fitted with an ultraviolet inverted phase C 18 Supelco® column (length
27 250 mm, internal diameter 2.1 mm), coupled to a 996 Waters® UV photodiode array

1 detector, after extraction by dichloromethane/acetone (50/50 v/v) using the Accelerated
2 Solvent Extracter Dionex® ASE 200. Chemical analyses were made in triplicate.

3

4 *2.2. Experimental design*

5

6 The polluted soil was mixed with the control soil at 1% concentration (1 part
7 polluted soil, 100 parts control soil). This was shown to be the level at which the polluted
8 soil was still strongly repellent to *F. candida* without being detrimental to its survival and
9 reproduction (Martínez Aldaya et al., 2005). Mixing of the two soils was done on moist soil
10 immediately after thawing, then the mixture was distributed among experimental boxes.
11 Boxes, made of crystal polystyrene, were cylindrical, 5 cm diameter and 3 cm height, with
12 cover lid. They were filled with 40 mL of the soil mixture, then they were kept in darkness
13 at 20°C. Moisture was kept constant by adding weekly distilled water. Twenty-five boxes
14 were prepared, 15 for microbial degradation, 10 others for zero-time analyses. At the end
15 of the incubation week (zero-time), 5 boxes were collected for microbiological analyses,
16 and 5 boxes were collected for chemical analyses. The duration of the experiment was
17 eight weeks after the incubation week.

18

19 *2.3. Microbiological analyses*

20

21 Total bacteria and fungi were counted from soil samples by mixing 10 g of soil with
22 50 mL sterile Ringer solution (Ramsay, 1984) in a Waring blender for 1.5 min at high
23 speed. Aliquots (0.1 mL) of soil suspension were spread respectively on Nutrient Agar
24 diluted to 1/10 (Difco, Detroit, USA) and on Yeast Extract Glucose (Difco, Detroit, USA)
25 media, on the basis of three replicates per dilution and medium. Colonies were counted
26 after 7-day incubation at 30°C. Total culturable bacteria and fungi were expressed in
27 Colony Forming Units of dry soil (CFU.g⁻¹).

1

2 PAH-degrading microbiota were enumerated on sterile polypropylene-microplates
3 (Nunc, Nunclon Delta, Wiesbaden, Germany), according to the method of Stieber et al.
4 (1994), with 250 μ L of mineral salts medium in each well and one out of four possible
5 PAHs (anthracene, phenanthrene, fluorene, fluoranthene, pyrene), regarded as most
6 representative of coal tar from the coke site, as sole carbon and energy source. Each well
7 was inoculated with 25 μ L of previously diluted soil suspension. Three wells were
8 inoculated with the same diluted soil suspension. The microplates were inoculated at 20°C
9 and incubated for 10-30 days then evaluated for coloured products. Enumeration of
10 specific PAH-degrading microorganisms was carried out using the Most Probable
11 Numbers method (de Man, 1977).

12

13 *2.4. Avoidance test*

14

15 Avoidance tests were performed in sterile crystal polystyrene Petri dishes (55 mm
16 diameter, 10 mm height), the bottom of which was lined with two half-disks of glass fiber
17 filter paper (50 mm diameter). The entire surface of each half-disk was covered with a soil
18 paste. One control half disk was covered with the control soil, the other with the polluted
19 soil diluted at 1%. The two half-disks were separated by a 2 mm space line, at the center
20 of which one individual of *F. candida* was deposited. The animals chosen for the
21 avoidance test were naive adults or subadults and came from the same batch culture,
22 which was maintained on fine quartz sand with ground cow dung as food for more than
23 two years (originating from one female collected in the Park of the Laboratory). The
24 position of the animal was recorded each 20 min up to 100 min. Previous assays showed
25 that 100 min were enough to let the animal choose definitely between both sides after
26 preliminary exploration of the Petri dish. Twenty replicates, in two successive batches of
27 ten, were followed together. During the experiment, Petri dishes were placed under a

1 Sharp® fluorescent illuminator in a chamber at 20°C. Care was taken that the animals
2 were not disturbed by the observer, who checked for the position of the animal through
3 the cover lid by help of a hand-held magnifying glass. Blank experiments using control soil
4 at both sides checked for the absence of any light gradient which could bias the results
5 (Salmon and Ponge, 1998). Totals of five counts over 100 min for each Petri dish were
6 used as scores for testing differences between control and polluted sides.

7

8 The repulsion of the contaminated soil turned to attraction after two-month
9 incubation. This unexpected result was checked by repeating the avoidance test the day
10 after the main test, using another aliquot of the contaminated soil and another batch of 20
11 naive animals issuing from the same culture.

12

13 *2.5. Statistical treatment of the data*

14

15 Choice in favour of the contaminated soil or of the control soil, before and after
16 incubation, was tested using sign tests, with the 20 Petri dishes as replicates and total
17 counts on both sides of Petri dishes as data.

18

19 Differences between treatments (T₀, T₊₂) were tested on microbiological and
20 chemical data by one-way ANOVA followed by a posteriori tests for differences among
21 means (Student-Newman-Keuls procedure). Data were log-transformed when necessary.

22

23 **3. Results**

24

25 *3.1. Chemical data*

26

1 Chemical characteristics of the soil samples (texture, moisture, pH, Total Organic
2 Carbon, Total Organic Nitrogen, Total Phosphorus, 16 US EPA PAHs) are reported in
3 Table 1. Metal concentrations of polluted and control soils are presented in Table 2.
4 Before use in our experiments both soils had been kept frozen at -20°C, at approximately
5 20% moisture.

6
7 Total cyanides were weakly present in the polluted soil, never exceeding 1 mg.kg⁻¹
8 dry soil (Table 1) and trace elements were of the same order as the geochemical
9 background (Table 2). The studied matrix (polluted soil diluted to 1/100 in the control soil)
10 contained about 10 mg.kg⁻¹ dry matter from the 16 US EPA PAHs (Table 3), with a
11 majority of 4-ring (65%) and a lesser amount of 3-ring (17%) PAHs (Fig. 1). Fluoranthene
12 was the best represented 4-ring PAH (about 51% of this category), followed by pyrene
13 (23%). Phenanthrene was the most represented 3-ring PAH (58%), followed by
14 anthracene (30%).

15
16 After two-month incubation, we observed a 50% decrease of PAH concentration
17 (Table 3). This decrease concerned 3- and 4-ring PAHs, the dominant forms, their rate of
18 disappearance being 50% and 65%, respectively (Fig. 1). Among 3-ring PAHs, most
19 reduction was displayed by phenanthrene (55%) and anthracene (66%). Among 4-ring
20 PAHs, most reduction was displayed by fluoranthene (68%) and pyrene (73 %).

21 22 *3.2. Microbiological data*

23
24 Total counts for soil samples of culturable bacteria and fungi are reported on
25 Figure 2. Colony-forming culturable microbiota were abundant and their counts remained
26 of the same order of magnitude before and after 2-month incubation, 10⁷ to 10⁸ bacteria
27 and 10⁵ to 10⁶ fungi per g dry soil, despite a weak but significant decrease.

1

2 Microbiota degrading 3- and 4-ring PAHs, and in particularly phenanthrene,
3 anthracene, fluorene and fluoranthene, which were present in large amounts in the initial
4 matrix, did not show any significant decrease (Fig. 3). On the contrary, pyrene-degrading
5 microflora (10^4 bacteria per g of dry soil in the initial matrix) showed a significant 100-fold
6 increase.

7

8 *3.3. Avoidance of the contaminated soil*

9

10 Previous to the incubation, the contaminated soil proved to be strongly repellent to
11 *Folsomia candida*, which shifted to the control soil in choice experiments (Fig. 4). After
12 two-month incubation, the direction of the choice was inverted, the percent choice in
13 favour of the contaminated soil being 85%. The test was repeated, in order to provide a
14 more convincing evidence of the attractive power of the contaminated soil after 2-month
15 incubation. The percent choice in favour of the contaminated soil was 88% in the repeat
16 test ($P = 2 \cdot 10^{-3}$).

17

18 **4. Discussion**

19

20 The contaminated soil used in the present study was heavily polluted with
21 hydrocarbons, among them the 16 PAHs of the EPA list amounted to 3‰ of its dry weight
22 (Table 1). After dilution with the control soil, the amount was still 10 times that of the
23 unpolluted control soil. According to the review by Bouchez et al. (1996) the composition
24 of the contaminated soil was typical of a pyrolysis product, by the dominance of
25 fluoranthene and pyrene (Table 3) and the narrow ratio phenanthrene/anthracene (1.9). In
26 two months, 50% of the global content in the 16 PAHs was degraded. This concerned
27 mainly 4-ring PAHs, which were the best represented compounds in the studied soil.

1 Three-ring PAHs also contributed to the observed decrease, but to a lesser extent. The
2 disappearance of PAHs was linked to bacterial degrading activity. Microbiota degrading 3-
3 and 4-ring PAHs, in particular phenanthrene, anthracene, fluoranthene and pyrene, were
4 found in large amounts, about 10^5 to 10^6 bacteria per g of dry soil (Fig. 3). This
5 demonstrates the occurrence of adapted microbiota in the coke site, probably because of
6 the ancient past of the site (Kästner et al., 1994).

7

8 *Folsomia candida* was strongly repelled by the contaminated substrate, even at
9 1% dilution in the control (unpolluted) soil, and was attracted to it after two-month
10 incubation in the presence of an indigenous microflora. We interpreted results of choice
11 experiments in terms of avoidance or attraction for a test (contaminated) soil, compared
12 with an uncontaminated soil used as a control. As in every choice experiment, attraction
13 and avoidance are two faces of the same biological property: the proneness of animals to
14 move among a variety of micro-sites which they encounter in the course of their
15 peregrination, their directional movements being dictated by the balance between
16 attraction and repellence. Traces of volatile and non-volatile molecules produced in the
17 environment of soil animals (or by the animals themselves) have been shown to elicit
18 attraction or avoidance, in particular fungal metabolites (Hedlund et al., 1995), earthworm
19 excreta (Salmon and Ponge, 2001) and pheromones (Verhoef, 1984). Movements of
20 Collembola far from or towards a fungal colony are highly correlated with the nutritional
21 value or toxicity of the strain (Sadaka-Laulan et al., 1998). In the present experiment, we
22 can hypothesize that physiological analogues of fungal metabolites, resulting from the
23 microbial degradation of PAHs, could be responsible for the attraction observed after 2-
24 month incubation of the contaminated soil.

25

26 In the absence of reliable methods for the analytical assessment of the
27 bioavailable fraction of PAHs in the soil, biological tests using soil animals may help to

1 directly address the environmental impact of pollution by hydrocarbons, and the extent to
2 which bioremediation will alleviate soil toxicity (Henner et al., 1999; Johnson et al., 2002).
3 The avoidance test used in the present study is a rapid, cheap assessment of the
4 behaviour of a test animal face to environmental pollution. Previous studies showed that it
5 could detect the presence of a repellent pollutant at a very low concentration, far below
6 toxicity thresholds (Martínez Aldaya et al., 2005). Other studies showed that avoidance
7 behaviour in soil invertebrates was strongly related with inadequacy of food or substrate to
8 sustain the growth of populations (Sadaka-Laulan et al., 1998; Shakir Hanna and Weaver,
9 2002). Attraction, reinforced by contact and aggregation pheromones, is an efficient way
10 by which soil animals find proper sites for feeding, moulting and ovipositing (Leonard and
11 Bradbury, 1984; Michelozzi et al., 1997; Salmon and Ponge, 2001). Thus, any shift from
12 avoidance to attraction (or neutral behaviour) of a polluted substrate might give an early
13 indication of potential changes in soil animal communities which could stem from
14 decontamination.

15

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17

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20

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Table 1. Physical and chemical characteristics of the polluted soil and the corresponding control soil, before use in the experiments. Means of three replicated measures are followed by standard deviations. Concentrations are expressed on a dry soil basis

	Texture	Moisture (%)	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)
Polluted soil	Silty sand	24.3±0.5	7.9±0.02	9	1700	620	2894±54	0.8
Control soil	Medium silt	6.4±0.1	8.4±0.04	0.7	670	410	0.97±0.09	<0.1

1

2

Table 2. Concentration of heavy metals ($\text{mg}\cdot\text{kg}^{-1}$ of dry soil) in the polluted soil compared with the geochemical background (Sterckeman *et al.* 2000). Means of three replicated measures are followed by standard deviations

	As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Polluted 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

1

2

Table 3. PAH concentrations (mg.kg^{-1} of dry soil) of the polluted soil, diluted in the control soil at 1% , before and after the 2-month experiment. Values are means of five replicates (\pm S.E.), followed by letters indicating homogeneous groups (Newman-Keuls test after ANOVA). <d.l. = below detection limit

	T0	T+2
Naphthalene	0.33 \pm 0.02b	0.39 \pm 0.01 a
Acenaphthylene	<d.l.	<d.l.
Acenaphthene	<d.l.	<d.l.
Fluorene	0.20 \pm 0.01 a	0.10 \pm 0.01 b
Phenanthrene	1.07 \pm 0.03a	0.49 \pm 0.05b
Anthracene	0.55 \pm 0.02a	0.13 \pm 0.01 b
Fluoranthrene	3.48 \pm 0.19a	0.90 \pm 0.09b
Pyrene	1.88 \pm 0.11 a	0.59 \pm 0.06b
Benzo(a)anthracene	0.74 \pm 0.05a	0.46 \pm 0.04b
Chrysene	0.67 \pm 0.04a	0.43 \pm 0.04b
Benzo(b)fluoranthrene	<d.l.	<d.l.
Benzo(k)fluoranthrene	0.24 \pm 0.01 a	0.22 \pm 0.02a
Benzo(a)pyrene	0.48 \pm 0.03a	0.39 \pm 0.04b
Dibenzo(a,h)anthracene	0.07 \pm 0.00b	0.26 \pm 0.01 a
Benzo(ghi)perylene	0.32 \pm 0.01b	0.40 \pm 0.03a
Indeno(123cd)pyrene	0.32 \pm 0.02a	0.28 \pm 0.03a
Σ 16 PAHs		

1

2

1 **Figure captions**

2

3 **Fig. 1.** Mean concentration of 2- to 6-ring PAHs, before and after 2-month incubation.

4 Values are means of five replicates. Differences among means were tested by
5 Student-Newman-Keuls (SNK) procedure following one-way ANOVA. Common
6 letters indicate homogeneous groups of the five PAH types, which were treated
7 separately

8

9 **Fig. 2.** Total (bacterial and fungal) microbiota in polluted soil before (To) and after 2-

10 month incubation (T+2). Differences among means were tested by Student-
11 Newman-Keuls (SNK) procedure following one-way ANOVA. Common letters
12 indicate bacterial and fungal homogeneous groups, which were treated separately

13

14 **Fig. 3.** PAH-degrading bacterial in polluted soil before (To) and after 2-month incubation

15 (T+2). Differences among means were tested by Student-Newman-Keuls (SNK)
16 procedure following one-way ANOVA. Common letters indicate bacterial PAH-
17 degrading homogeneous groups, which were treated separately. Phe =
18 phenanthrene, Flu = fluoranthene, Ant = anthracene, Flt = fluoranthene, Pyr =
19 pyrene

20

21 **Fig. 4.** Avoidance/attraction test. Percent choice in favour of the control (unpolluted) or

22 test soil (1% dilution of the polluted soil) by *Folsomia candida*, before (To) and
23 after 2-month incubation (T+2) of the polluted substrate. Significance of the
24 difference between control and test side was assessed by a sign test (20
25 replicates)

26

Table 1. Physical and chemical characteristics of the polluted soil and the corresponding control soil, before use in the experiments. Means of three replicated measures are followed by standard deviations. Concentrations are expressed on a dry soil basis

	Texture	Moisture (%)	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)
Polluted soil	Silty sand	24.3±0.5	7.9±0.02	9	1700	620	2894±54	0.8
Control soil	Medium silt	6.4±0.1	8.4±0.04	0.7	670	410	0.97±0.09	<0.1

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Table 2. Concentration of heavy metals ($\text{mg}\cdot\text{kg}^{-1}$ of dry soil) in the polluted soil compared with the geochemical background (Sterckeman *et al.* 2000). Means of three replicated measures are followed by standard deviations

	As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Polluted 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

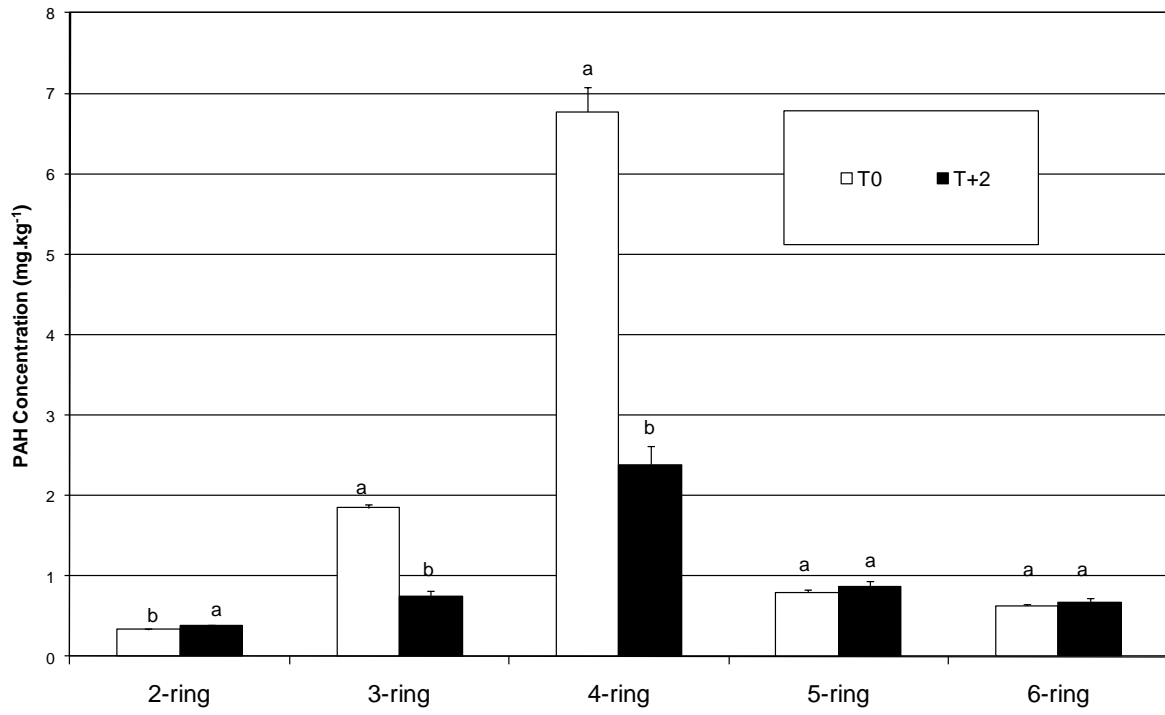
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Table 3. PAH concentrations ($\text{mg}\cdot\text{kg}^{-1}$ of dry soil) of the polluted soil, diluted in the control soil at 1%, before and after the 2-month experiment. Values are means of five replicates (\pm S.E.), followed by letters indicating homogeneous groups (Newman-Keuls test after ANOVA). <d.l. = below detection limit

	T0	T+2
Naphthalene	0.33 \pm 0.02b	0.39 \pm 0.01a
Acenaphthylene	<d.l.	<d.l.
Acenaphthene	<d.l.	<d.l.
Fluorene	0.20 \pm 0.01a	0.10 \pm 0.01b
Phenanthrene	1.07 \pm 0.03a	0.49 \pm 0.05b
Anthracene	0.55 \pm 0.02a	0.13 \pm 0.01b
Fluoranthrene	3.48 \pm 0.19a	0.90 \pm 0.09b
Pyrene	1.88 \pm 0.11a	0.59 \pm 0.06b
Benzo(a)anthracene	0.74 \pm 0.05a	0.46 \pm 0.04b
Chrysene	0.67 \pm 0.04a	0.43 \pm 0.04b
Benzo(b)fluoranthrene	<d.l.	<d.l.
Benzo(k)fluoranthrene	0.24 \pm 0.01a	0.22 \pm 0.02a
Benzo(a)pyrene	0.48 \pm 0.03a	0.39 \pm 0.04b
Dibenzo(a,h)anthracene	0.07 \pm 0.00b	0.26 \pm 0.01a
Benzo(ghi)perylene	0.32 \pm 0.01b	0.40 \pm 0.03a
Indeno(123cd)pyrene	0.32 \pm 0.02a	0.28 \pm 0.03a
1 Σ 16 PAHs	10.39\pm0.55a	5.07\pm0.43b

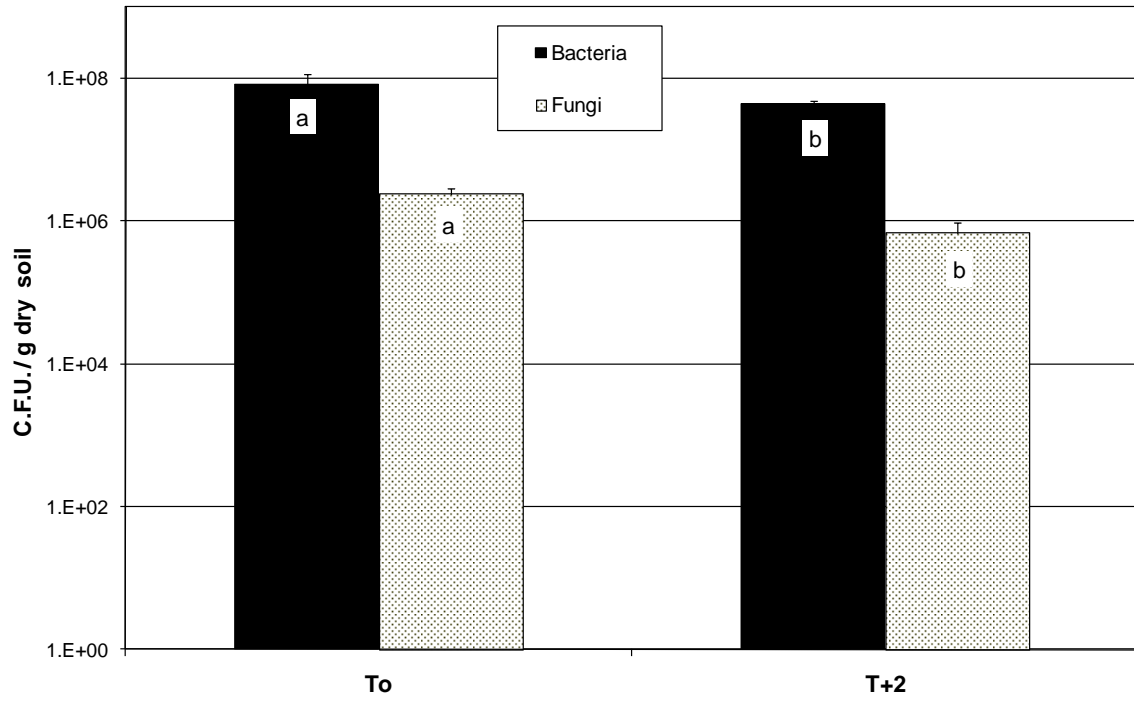
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2 Fig. 1

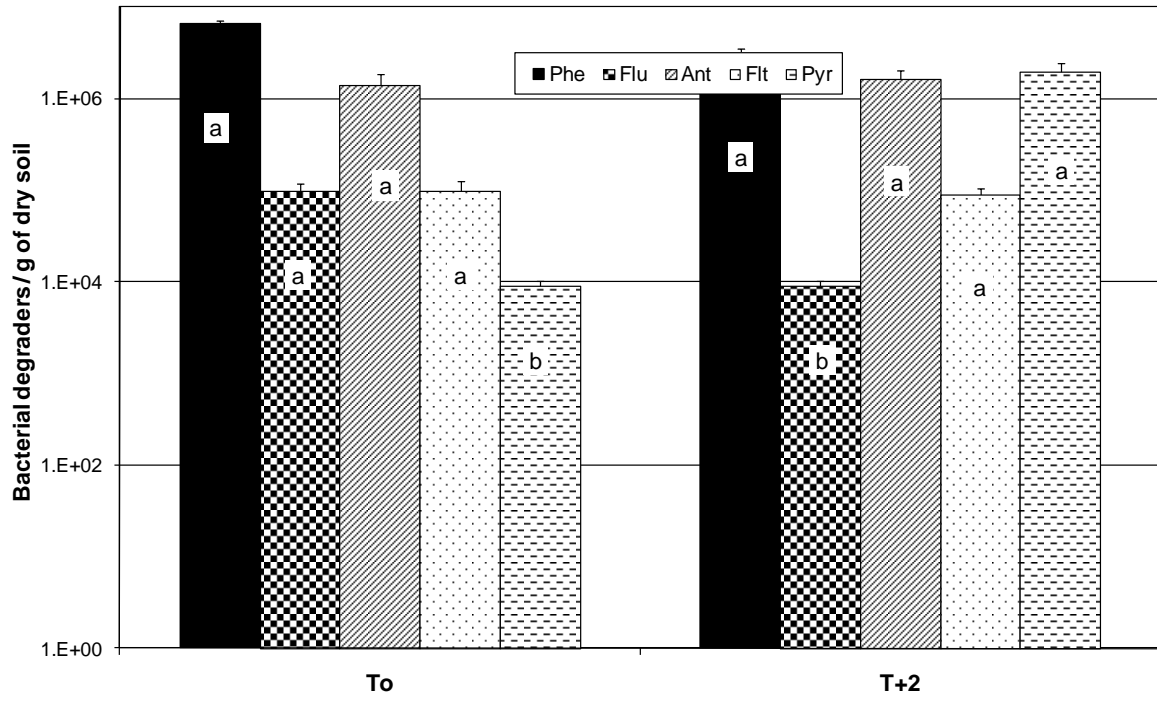
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2 Fig. 2

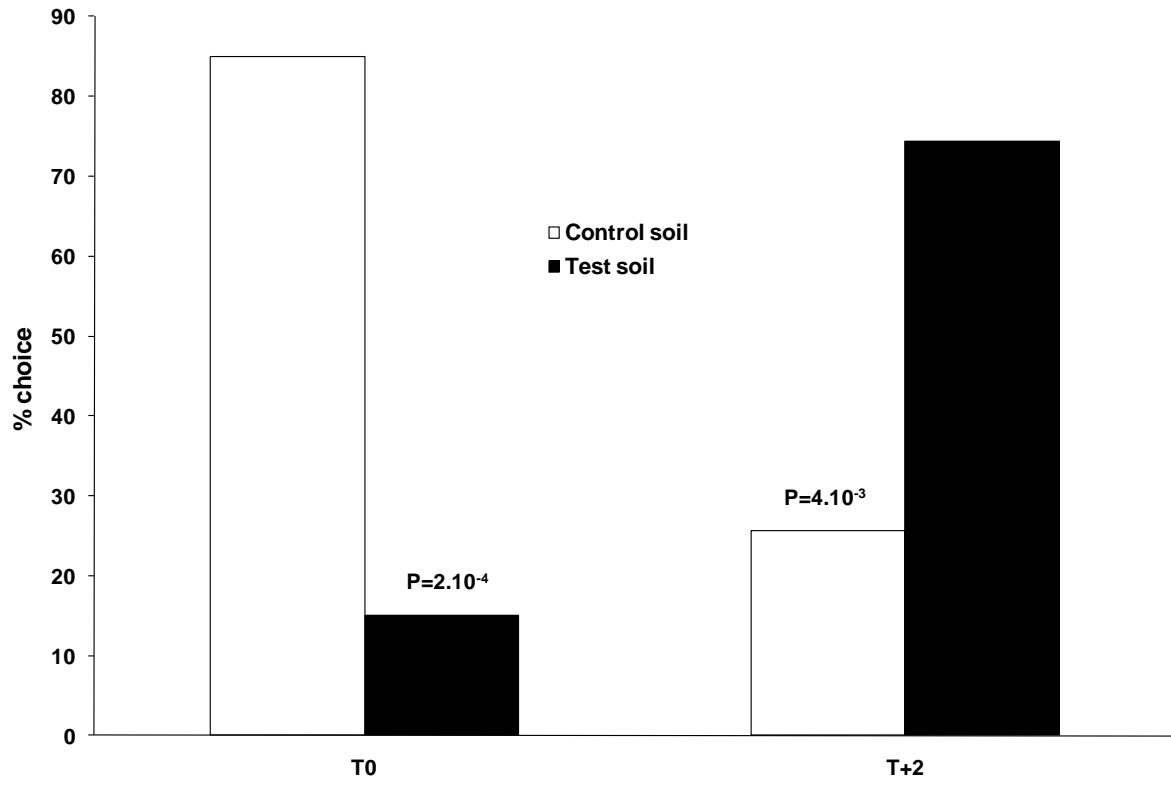
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2 Fig. 3

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2 Fig. 4

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