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Abstract

An avoidance test using the soil springtail *Folsomia candida* was used to assess changes in contamination levels at low doses of PAHs following incubation with indigenous microflora. A soil from a former coke site was diluted to 1% in an unpolluted soil from the same site, which was used as a control, then both substrates were remoistened to 80% field capacity. The diluted soil was previously shown to be strongly repellent to *F. candida*, although not toxic. After two-month incubation at 20°C, the mixture lost its repellence capacity and became attractive to the test animal, while the global 16 PAHs content had decreased to a great extent (50%). PAH disappearance was linked to the occurrence of indigenous microbiota able to degrade hydrocarbons.

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Substrate used for the experiment and chemical analyses

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

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

2.2. Experimental design

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

2.3. Microbiological analyses

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

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

2.4. Avoidance test

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

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

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

2.5. Statistical treatment of the data

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

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

3. Results

3.1. Chemical data

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

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

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

3.2. Microbiological data

Total counts for soil samples of culturable bacteria and fungi are reported on Figure 2. Colony-forming culturable microbiota were abundant and their counts remained of the same order of magnitude before and after 2-month incubation, 10^7 to 10^8 bacteria and 10^5 to 10^6 fungi per g dry soil, despite a weak but significant decrease.

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

3.3. Avoidance of the contaminated soil

Previous to the incubation, the contaminated soil proved to be strongly repellent to *Folsomia candida*, which shifted to the control soil in choice experiments (Fig. 4). After two-month incubation, the direction of the choice was inverted, the percent choice in favour of the contaminated soil being 85%. The test was repeated, in order to provide a more convincing evidence of the attractive power of the contaminated soil after 2-month incubation. The percent choice in favour of the contaminated soil was 88% in the repeat test ($P = 2.10^{-3}$).

4. Discussion

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

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

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

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

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

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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

Table 2. Concentration of heavy metals (mg.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 (± S.E.), followed by letters indicating homogeneous groups (Newman-Keuls test after ANOVA). <d.l. = below detection limit

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

Figure captions

Fig. 1. Mean concentration of 2- to 6-ring PAHs, before and after 2-month incubation. Values are means of five replicates. Differences among means were tested by Student-Newman-Keuls (SNK) procedure following one-way ANOVA. Common letters indicate homogeneous groups of the five PAH types, which were treated separately

Fig. 2. Total (bacterial and fungal) microbiota in polluted soil before (To) and after 2-month incubation (T+2). Differences among means were tested by Student-Newman-Keuls (SNK) procedure following one-way ANOVA. Common letters indicate bacterial and fungal homogeneous groups, which were treated separately

Fig. 3. PAH-degrading bacterial in polluted soil before (To) and after 2-month incubation (T+2). Differences among means were tested by Student-Newman-Keuls (SNK) procedure following one-way ANOVA. Common letters indicate bacterial PAH-degrading homogeneous groups, which were treated separately. Phe = phenanthrene, Flu = fluoranthene, Ant = anthracene, Flt = fluoranthene, Pyr = pyrene

Fig. 4. Avoidance/attraction test. Percent choice in favour of the control (unpolluted) or test soil (1% dilution of the polluted soil) by *Folsomia candida*, before (To) and after 2-month incubation (T+2) of the polluted substrate. Significance of the difference between control and test side was assessed by a sign test (20 replicates)

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 ($mg.kg^{-1}$)**
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2

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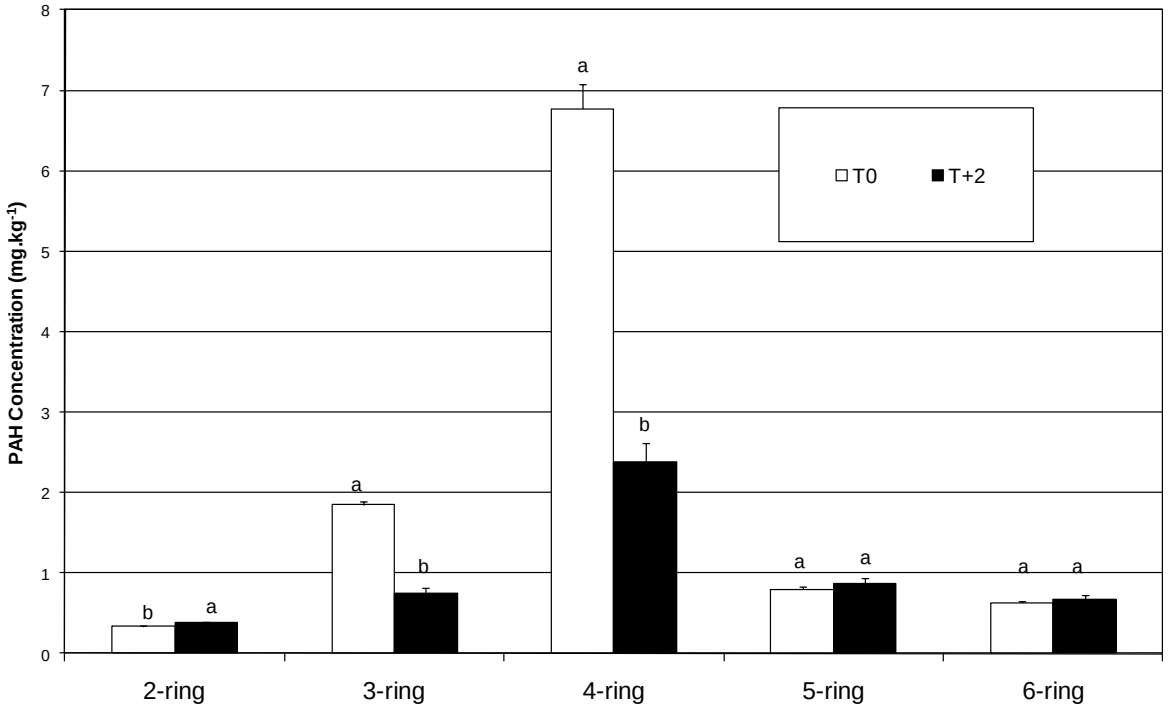
Anthracene	0.55±0.02a	0.13±0.01b
Fluoranthrene	3.48±0.19a	0.90±0.09b
Pyrene	1.88±0.11a	0.59±0.06b
Benzo(a)anthracene	0.74±0.05a	0.46±0.04b
Chrysene	0.67±0.04a	0.43±0.04b
Benzo(b)fluoranthrene	<d.l.	<d.l.
Benzo(k)fluoranthrene	0.24±0.01a	0.22±0.02a
Benzo(a)pyrene	0.48±0.03a	0.39±0.04b
Dibenzo(a,h)anthracene	0.07±0.00b	0.26±0.01a
Benzo(ghi)perylene	0.32±0.01b	0.40±0.03a
Indeno(123cd)pyrene	0.32±0.02a	0.28±0.03a
Σ 16 PAHs	**10.39±0.55a**	**5.07±0.43b**


PAH Concentration (mg.kg-1)
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1

2 Fig. 1

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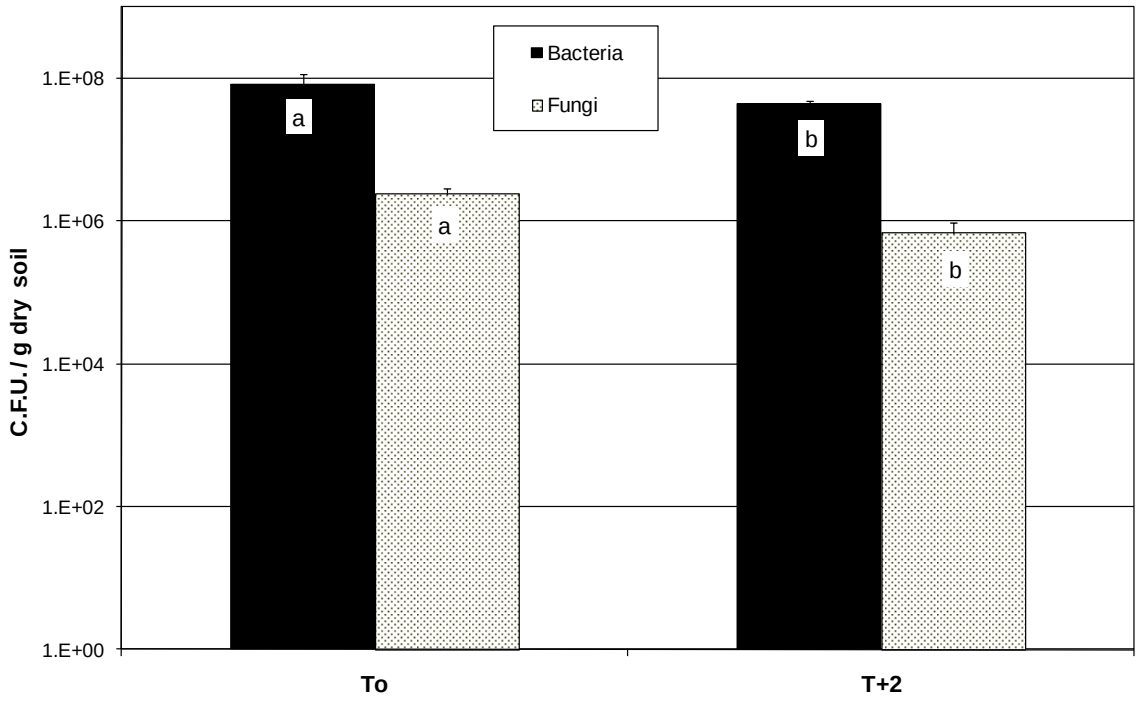
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2 Fig. 2

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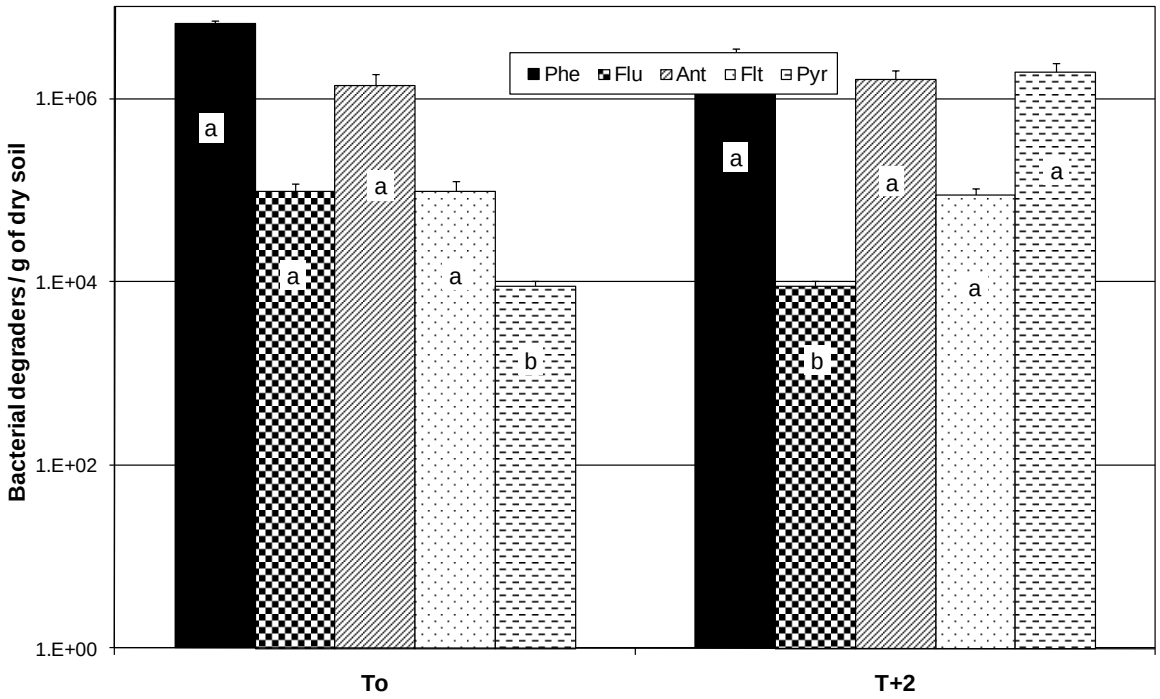
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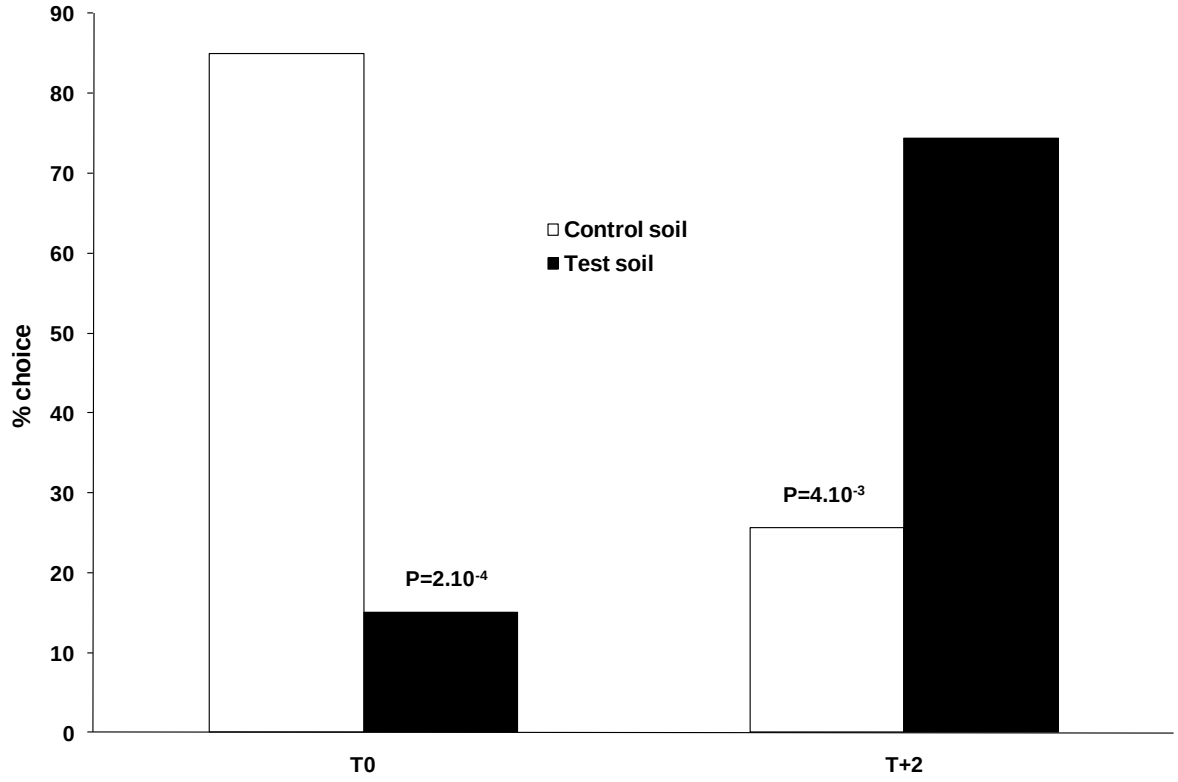
Flu
Ant
Flt
Pyr
Bacterial degraders / g of dry soil
1.E+06
1.E+04
1.E+02
1.E+00
a
a
a
a
b
a
b
a
a
a
To
T+2

Fig. 3


90
80
70
60
50
40
30
20
10
0
% choice
Control soil
Test soil
P=2.10-4
P=4.10-3
T0
T+2

1

2 Fig. 4

3