Investigating the effect of *Medicago sativa* L. and *Trifolium pratense* L. root exudates on PAHs bioremediation in an aged-contaminated soil.

Marie Davin*[a,b], Amandine Starren[a], Emilie Marit[a], Kévin Lefébure[a], Marie-Laure Fauconnier[b,c]& Gilles Colinet[a,c]

[a] Soil-Water-Plant Exchanges, University of Liège, Gembloux Agro-Bio Tech, 2 Passage des Déportés, 5030 Gembloux, Belgium

[b] Laboratory of Chemistry of Natural Molecules, University of Liège, Gembloux Agro-Bio Tech, 2 Passage des Déportés, 5030 Gembloux, Belgium

[c] These authors contributed equally to this work.

* corresponding author information:
ORCID ID: 0000-0003-3899-4218
mdavin@uliege.be or mariedavin@gmail.com
+3281622209 or +3281622290

Abstract

Polycyclic aromatic hydrocarbons (PAH) are persistent organic compounds of major concern that accumulate in the environment, especially soils, and require remediation. Researches to develop bioremediation and phytoremediation (alternative eco-friendly technologies) are being conducted. First a bioaccessibility measurement protocol was adapted to a brownfield soil using Tenax® beads in order to compare PAHs bioaccessibility in soil samples. PAHs desorption kinetics were established, described by a site distribution model, and a common extraction time was calculated (48 h). Second the role of two *Fabaceae* (*Medicago sativa* L. or *Trifolium pratense* L.) root exudates in enhancing PAHs bioaccessibility and biodegradation in the studied soil was evaluated during microcosms experiments (28°C). The CO_2 emissions were significantly higher in presence of *T. pratense* exudates; the dehydrogenase activities showed improvements of the soil microbial activity in presence of two types of root exudates compared to untreated soil samples; the PAHs residual contents decreased more in untreated samples than in the presence of *T. pratense* exudates; and *M. sativa* exudates lowered PAHs bioaccessibility but not residual contents.

Keywords: PAH; Tenax® extraction; bioaccessibility; bioremediation; brownfield soil; plant root exudates

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are composed of two or more condensed aromatic rings and are usually classified in three main categories: light PAHs of three rings or less, intermediate PAHs of four rings and heavy PAHs of four rings or more (INERIS 2005). These ubiquitous organic compounds are naturally brought into the environment through diagenetic, petrogenetic or pyrolytic processes, but the major source remains incomplete combustions of natural (i.e. volcanic eruptions), and mostly anthropogenic origin such as industrial manufacturing, fuel combustions, or waste disposal. PAHs become more hydrophobic as the number of aromatic cycles raises. Therefore once emitted in the environment, PAHs tend to sorb to solid particles, which renders them less susceptible to biotic and abiotic degradation, and therefore more persistent (Yu et al., 2018). PAHs health-concerning properties are real threats towards ecosystems and motivate the need to develop remediation strategies and control tools.

Over the last decades, the interest in the use of environmental friendly and cost-effective soil remediation techniques has largely increased (Alegbeleye et al., 2017). The use of living microorganisms or plants to dissipate soil pollution is often summarized as *bioremediation* and *phytoremediation* technologies, respectively (Ouvrard et al., 2013). However those techniques can hardly be considered separately as microorganisms and plants closely interact at the soil's solid, liquid and gaseous interfaces. It is indeed now well-acknowledged that plant roots create favorable conditions for microorganisms in their immediate proximity (2 mm), which is named the *rhizospheric effect* (Martin et al., 2014), but also that plant-microbe associations can be beneficial to the plants (Uroz et al., 2019).

Besides favoring the microbial community, studies have shown that the presence of plants also improved PAHs dissipation in contaminated soil. This includes members of the *Fabaceae* family (Wei and Pan, 2010; Hamdi et al. 2012; Alves et al., 2018). *Fabaceae* are good candidates for phytoremediation on brownfield soils because they are capable of colonizing hydrocarbon contaminated soils which often present very high carbon-nitrogen ratio (Hall et al. 2011). However the mechanisms through which plants enhance PAHs biodegradation in soil (i.e. rhizodegradation) are not yet fully understood.

Biodegradation processes are balanced by two major phenomena: (i) the mass transfer of a compound to a microbial cell and (ii) the uptake and metabolization of this compound by the living cell. The pollutant intrinsic physico-chemical properties (i.e. aqueous solubility, hydrophobicity, and molecular structure), along with environmental factors (such as content and nature of organic matter or clay minerals in soil), will influence the compound concentrations in the aqueous phase and thus their accessibility to degrading agents (microorganisms and their enzymes). Other factors such as pH, salinity, temperature, water content, mineral nutrients, redox potential, and water-dissolved oxygen will provide conditions more or less favourable to the microbial activity (Haritash and Kaushik 2009).

When it comes to rhizodegradation the general explanation found in the literature suggests that the enhanced dissipation of PAHs is caused by the rhizospheric effect, which itself is a combination of several physical and chemical phenomena: (i) increased contact between soil and microorganisms (Ouvrard et al., 2014), (ii) soil aeration, and (iii) the release of exudates by plant roots which provides the microbiota with easily accessible carbon sources and thus increases microbial communities (Alagić et al. 2015).

Indeed the majority of root exudates are composed of organic acids, sugars and amino acids. But studies about secondary plant metabolites in general have shown a large diversity of compounds that are released in the environment, some of which exhibit tensioactive (or surfactant) properties due to an amphiphilic nature. Such compounds are very often heterosides,

(a hydrophobic skeleton of steroidal or triterpenoidal nature coupled to a glycose (hydrophilic) moiety), and commonly referred to as saponins. Such compounds have been detected in members of the *Fabaceae* family (Vincken et al. 2007; Kregiel et al. 2017). Surfactants can place themselves at the interface between a hydrophobic and a hydrophilic phase and have been the subject of soil remediation studies, either in "washing technologies" (Von Lau et al. 2014) or to enhance mass transfer of contaminants towards degrading microorganisms (Kobayashi et al. 2012).

Based on this literature, a study was designed to determine the role of root exudates from two *Fabaceae* (*Medicago sativa* L. or *Trifolium pratense* L.) in enhancing PAHs bioaccessibility as part of the *rhizospheric effect*.

Hydrophobic organic compounds (HOCs) bioavailability/bioaccessibility has been intensively discussed (Ehlers and Luthy 2003; Semple et al. 2004) and will not be reminded here. However, the scientific community agrees that the fraction of a contamination that is the most likely to be degraded by the soil microbiota will be accessed in the aqueous phase. That fraction is named "bioaccessible", according to Semple et al. (2003) (i.e. "the compound that is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical"). Therefore analytical developments have been oriented to give the closest representation of the HOCs fraction that is bioaccessible to microorganisms in order to evaluate the potential for bioremediation of a given soil (Semple et al. 2003).

Cornelissen et al. (1997) developed a solid-phase extraction technique using Tenax® beads that mimic the interaction between the contaminants and the microbiota in the aqueous phase, if all the bioaccessible contaminants were degraded by these organisms. The determination of the accessible fraction of a contamination is however directly related to the time of contact between the microbial surrogate (the Tenax® beads) and the aqueous phase of a soil, and therefore desorption kinetics of a compound in a contaminated soil must be established to determine a minimal time of contact. As this time of extraction must be representative of a compound's bioaccessibility, it also should be economically affordable and cost-effective if the analytical method is to be applied routinely (for example to monitor the bioaccessibility of a pollutant in a soil, whether a specific treatment is applied or not).

Several models have been used by searchers to describe HOCs, and more specifically PAHs, desorption kinetics from soils. It is generally admitted that PAHs desorption occurs in several stages (Richardson and Aitken 2011). In an attempt to simplify descriptions, the compartment model is often used to reduce the phenomenon to a few representative stages described by first-order kinetics. The first stage is the rapid release of the most accessible fraction (F_{rap}) of the PAHs and is assimilated to the fraction that could be degraded by microorganisms. Another model, the site distribution model (first suggested by Connaughton et al. 1993) is based on a gamma distribution of rate coefficients, and considers the system as a continuum of compartments. While the use of this model does not allow to properly quantify rapidly and slowly desorbing fractions, it is probably more representative of the actual processes than the compartment model.

To evaluate the role of root exudates on the PAHs bioaccessible fraction, the first step of the present study was to adapt a bioaccessibility measurement protocol to the studied contaminated soil. Therefore, desorption kinetics of PAHs in the studied soil were determined and described using a model. Afterwards, a common and cost-effective Tenax® beads extraction time was established as a comparison basis for PAHs bioaccessibility assessments. In a second time, contaminated soil was incubated in presence of plant-root exudates in an attempt to enhance PAHs bioaccessibility. Two types of exudates and two incubation periods were tested while several parameters were examined: (i) the carbon dioxide emission was monitored during the incubation process to assess for microbial activity; (ii) dehydrogenase activity was determined

at the end of each incubation period as an indicator of the soil microbial activity; (iii) the residual PAHs contents and (iv) the bioaccessible PAHs were determined on soil samples after each incubation period to evaluate the impact of plant-root exudates on PAHs dissipation and bioaccessibility.

2. MATERIALS AND METHODS

2.1. *Soil material*

The experimental aged-contaminated soil has already been described in a former study (Davin et al., 2018) but its characteristics will be reminded hereunder. The soil was sampled from a brownfield in Saint-Ghislain (Belgium) in a former coking plant and has been exposed for 70 years to petroleum hydrocarbons, PAHs, cyanides and trace elements. Particle size distribution (81.1 % sand, 10.7 % silt, 8.2 % clay) identified the soil as loamy sand, pH was 6.7, total organic carbon was 9.44 ± 0.22 % (W/W), and total nitrogen content was 0.16 ± 0.02 % (W/W). Soil was sampled, allowed to dry at ambient air, sieved through a 2-mm sieve and stored in sealed boxes until further use. Before the experiments, the contents of 15 PAHs were determined to range from 2.9 ± 0.1 µg g^{-1} DW to 65.9 ± 7.1 µg g^{-1} DW (initial individual concentrations are in online resource 1). The studied PAHs are Acenaphtene (Ace), Anthracene (Anthr), Benzo(a)anthracene (BaA), Benzo(a)pyrene (BaP), Benzo(b)fluoranthene (BbF), Benzo(ghi)perylene (BghiP), Benzo(k)fluoranthene (BkF), Chrysene (Chrys), Dibenzo(ah)anthracene (DBahA), Fluoranthene (F), Fluorene (Fle), Indeno(123-c,d)pyrene (IcdP), Naphtalene (N), Phenanthrene (Phen), and Pyrene (Pyr).

2.2. *Plant root exudates: production and characterization*

Plant root exudates production was inspired by Louvel (2010). Seeds of *Medicago sativa* L. and of *Trifolium pratense* L. were purchased from Ecosem and presented a germination rate of over 95%. After surface sterilization in a 6% (w/v) solution of hydrogen peroxide for ten minutes, plants were grown on hydroponic floating devices; using Hoagland's nutritive solution (Hoagland and Arnon 1950). Air-blowers allowed proper oxygenation for the roots and plants were kept in a greenhouse where lamps assured 12h of light per day when necessary. Once a week, root-parts were rinsed of the nutritive solution and placed in 1 litre of distilled water for 5 hours. The aqueous solution was filtered on paper filter (11 µm), frozen and lyophilized. Remaining dry exudates were homogenized and stored at - 20°C until further use. All exudates were pooled together by plant type. The total organic carbon and the total nitrogen contents were respectively 11.37±0.22% and 0.868±0.016% (w/w) for *Medicago sativa* exudates (E_MS), and 10.46±0.22% and 0.984±0.016% (w/w) for *Trifolium pratense* exudates (E_TP).

2.3. *PAHs desorption kinetics*

Desorption kinetics was measured five times according to a method adapted from Cornelissen et al. (1997) and Barnier et al. (2014). Briefly, 2.0 g of soil were weighed into glass centrifuge tubes. 50 mL of 0.01 M $CaCl_2$ and 0.003 M NaN_3 were added as biocides along with 0.5 g of Tenax® beads (60-80 mesh). The tubes were shaken for 1, 2, 4, 8, 16, 24, 48, 72 or 96 hours on a rotary agitator (40 cycles min^{-1}). Tubes were then centrifuged (10 min; 2000 x g) to separate the soil from the Tenax® beads. The floating beads were separated by filtration on a Buchner vacuum device and air dried. Sorbed PAHs were recovered from Tenax® by a 60 min sonication with 20 mL of a 50:50 (V/V) n-hexane: acetone mixture, repeated twice. The organic phase was evaporated with a rotative evaporation device, and replaced with acetonitrile. The final acetonitrile extract was weighed for volume determination and analysed for PAHs. Each

PAH amount extracted by Tenax® beads was then used to calculate the remaining sorbed fraction in soil as follows

$$\frac{S_t}{S_0} = \frac{C_{tot_{in}} - C_{ext_t}}{C_{tot_{in}}} \quad (1)$$

where $C_{tot\ in}$ is the total initial PAH concentration in the soil [µg g^{-1} DW]; $C_{ext\ t}$ is the amount of PAH adsorbed by Tenax ® beads after t hours of extraction [µg g^{-1} DW]; S_t is the sorbed fraction of compound remaining after t hours of extraction; and S_0 is the initial sorbed fraction, assumed to be the total initial PAH concentration.

2.4. *Incubation experiments*

Incubation experiments were conducted in microcosms according to AFNOR XP U44-163. Briefly, 15 g of dry soil were pre-incubated for 3 days at 80 % of water holding capacity. Once amendments were added to samples (day 0 of incubation), two vessels were placed next to each sample in a sealed jar. One vessel was filled with distilled water to prevent soil desiccation and one was filled with NaOH solution to control carbon dioxide emission. Exudates were added to soil samples in order to reach 5 mg g^{-1} DW, for both plant types. Untreated soil served as control and two incubation periods (14 and 28 days starting at the addition of exudates) were tested. All modalities were repeated four times for a total of 24 samples. All jars were sealed and incubated at 28°C, in the dark. At the end of the incubation period, soils were sacrificed for dry weight, dehydrogenase activity and PAHs measurements (residual and bioaccessible) concentrations. Results related to soil samples with 5 mg g^{-1} DW of *Medicago sativa* L. or *Trifolium pratense* L. exudates are named E_MS and E_TP, respectively. Results related to control samples are named C.

2.5. *Chemical analyses*

Dry weight determination.

Soil samples dry weight determination was based on ISO 11465:1993 cor 1994.

Total nitrogen content.

Total nitrogen determination was based on ISO 11261:1995.

Total organic carbon.

Total organic carbon determination was based on ISO 14235:1998.

Carbon dioxide emission.

Carbon dioxide emission was monitored for each soil sample throughout the whole incubation following AFNOR XP U44-163.

Dehydrogenase activity.

Dehydrogenase activity was measured for each soil sample after the incubation following a method described by Shaw and Burns (2005).

Bioaccessible PAHs determination in soil samples.

Bioaccessible PAHs determination in soil samples was realised on fresh soil samples as described in the PAHs desorption kinetics section, except the samples were agitated for 48 hours in the presence of the Tenax® beads (see section 3.1 for time choice).

Total PAHs determination in soil samples.

Total PAHs extraction in soil samples was based on ISO 13877:1998. The final acetonitrile extract was analysed for PAHs.

PAHs analysis.

PAHs were analysed in acetonitrile extracts of desorption kinetics, bioaccessible and residual samples based on ISO 13877:1998.

Models and statistics.

R 3.4.3 was used to generate PAHs desorption models. The Levenberg-Marquardt algorithm was used to minimize squared residuals between experimental and calculated values for each or the four tested models (Table 1) (Prague et al. 2012). A model was selected for each PAH using the Bayesian information criterion (BIC) which estimates the relative information of a model as follows

$$BIC = k.\ln(n) - 2.ln(L) \quad (2)$$

where k is the number of parameters of a model, n is the number of data points and L is the maximized value of a likelihood function. R function is BIC(model_iner2).

All statistical analyses related to the incubation experiment were carried out using Minitab 17.0. Equality of variances were verified according to Levene's test, data were analysed by general linear model or one-way analysis of variance and mean values were compared by Tukey's test at the 5 % confidence level.

3. RESULTS AND DISCUSSION

3.1. *Assessing PAHs bioaccessibility*

Modelling PAHs desorption kinetics.

Soil samples were extracted for increasing time steps in the presence of Tenax® beads and the recovered PAHs amounts were used to calculate remaining sorbed fractions for each extraction time according to equation (1) (data is available in online resource 1). Then modelling was used to describe desorption kinetics (Figure 1). BIC values were calculated using R for each tested model and are available in online resource 2. These values have no meaning by themselves and can only be used to compare models generated from a same data set. The smallest BIC value indicates the model that better represents the data set and was obtained by the site distribution model for all compounds except for the heaviest PAHs (DBahA, BghiP, IcdP) for which it was obtained by the first-order three-compartments model. These three compounds showed BIC-value differences of four to six units with the second-best model, which in each case was the site distribution model. According to Kass and Raftery (1995) this range of difference of BIC value between models is positive, but not strong. Therefore, to homogenize the description of desorption kinetics, the site distribution model was chosen for all compounds (Figure 1).

PAHs desorption parameters.

Desorption models were used to determine a minimal extraction time (t_{ex}) for bioaccessibility measurement of each PAH. This t_{ex} should represent the time for the most accessible fraction to equilibrate with Tenax® beads. Therefore, t_{ex} values were calculated as the time for which the slope to the desorption model closes down to zero. Given the asymptotic nature of the models, the slope limit was arbitrarily set to 10^{-3} and successive approximations were made according to the following equation

$$\frac{y_{t_{ex}-24}-y_{t_{ex}}}{24} \leq 0.001 \quad (3)$$

where y is the calculated value of a PAH site distribution equation at different times; and t_{ex} is the extraction time [h].

Calculated t_{ex} values and site distribution models parameters (alpha and beta) are presented in Table 2. Alpha values range from $6.88.10^{-3}$ to $1.14.10^{-2}$, beta values range from $8.98.10^{-4}$ h to 1.34 h, and calculated extraction times are either 24 h (for the lightest PAHs) or 48 h. Thus a common 48 h extraction time was used to determine PAHs bioaccessible contents in the incubation experiment. Let us stress here that the "bioaccessible contents" that will be discussed further down actually are "contents that are extracted after 48 h of presence of Tenax® beads."

3.2. *PAHs bioremediation in presence of root exudates*

Respiration curves and dehydrogenase activities.

Figure 2 presents CO_2 emissions of (un)treated soil samples throughout incubation in microcosms. Statistical analysis was performed after $\log_{10}$ transformation.

E_TP soil samples exhibit significantly higher cumulated CO_2 emissions than C and E_MS samples after 7, 21, and 28 days of incubation (p=0.000). E_MS however is never significantly different from C samples. Assuming that all the amendments added to E_TP and E_MS samples had been completely mineralized, CO_2 emissions would be of respectively 1.92 ± 0.04 and 2.08 ± 0.04 mg CO_2 g^{-1} DW. In the case of E_MS samples, the observed emission is lower than the calculated emission, but in the case of E_TP samples it is higher, suggesting that TP exudates influence CO_2 emissions to a greater extent than their own degradation, and also that MS exudates were not entirely mineralized.

Figure 3 shows (un)treated soil samples dehydrogenase activities before and after incubation. There is a significant interaction between time and treatment. C samples activities decrease throughout the incubation and are significantly lower after 28 days of incubation than at the beginning of the incubation. E_TP and E_MS samples, however, show increases after 14 days (respectively + 134% and + 99.5%) before lowering back during the last two weeks of incubation. Being an indicator of soil general health (Das and Varma 2011), the raise in this enzyme activity suggests that the amended exudates have no toxic effect towards the soil microbiota.

PAHs residual and bioaccessible contents.

Figure 4 and Figure 5 respectively show (un)treated soil samples residual and bioaccessible PAHs contents before and after incubation.

For both sets of results, PAHs contents were grouped to provide better information: 2-3 rings (N, Ace, Fle, Phen, and Anthr); intermediate 4 rings (F and Pyr); 4-6 rings (BaA, Chrys, BbF, BkF, BaP, DBahA, BghiP, and IcdP); and total PAHs (N to IcdP). Statistical analyses on bioaccessible contents were performed after $\log_{10}$ transformation. Significant differences

appear between the bioaccessible contents measured on the untreated soil to establish desorption kinetics and the bioaccessible contents measured after 3 days of pre-incubation (respectively named "-3 days" and "0 days" in Fig. 5). After this pre-incubation period, the bioaccessible contents are respectively three (2-3 rings PAHs), four (intermediate 4 rings PAHs), two (4-6 rings PAHs), and three (total PAHs) fold the ones measured initially in desorption kinetics.

Statistical analyses on both residual and bioaccessible contents show no interaction between time and treatment. Different behaviours appear within each treatment. (i) The residual content of 2-3 rings PAHs is significantly lower ($p<0.05$) for E_TP and E_MS samples and very significantly lower ($p<0.01$) for C samples after 14 days of incubation whilst the bioaccessible content of 2-3 rings PAHs is highly significantly lower ($p=0.000$) after 14 days of incubation for each treatment. PAHs could have been dissipated from the soil by biotic (such as biodegradation) or abiotic processes (such as volatilization, which would not come as a surprise for a volatile compound such as naphthalene with a vapor pressure of 10.5 Pa at 25°C). Regardless, this means the less sorbed fraction of light PAHs was eliminated from the soil, and was not replaced. So the remaining PAHs are more or less strongly sorbed to the soil and for this group of PAHs, the addition of TP or MS exudates does not enhance dissipation compared to untreated samples.

(ii) The residual content of intermediate 4 rings PAHs is significantly lower for E_TP samples after 14 days and for C samples after 28 days of incubation, whilst there is no significant lowering of this PAHs group in E_MS samples after 28 days. On the other hand, the bioaccessible sum of intermediate 4 rings PAHs is highly significantly lower ($p=0.000$) after 14 days of incubation for each treatment. The fact that this group of PAHs dissipates faster in E_TP than in C samples is probably caused by the addition of TP exudates that provided a more easily available source of carbon for the soil microbiota (Louvel 2010) and boosted its activity, allowing it to start degrading PAHs faster. In the case of E_MS samples though, the fact that this group of PAHs bioaccessibility lowers significantly whilst their residual content remains statistically unchanged suggests that MS exudates might be preventing PAHs to be dissipated by influencing their bioaccessibility.

(iii) The residual content of 4-6 rings PAHs does not significantly lower after 28 days of incubation for any treatment. As for the bioaccessible content of 4-6 rings PAHs, after being enhanced by the pre-incubation process, it lowers back towards the initial (-3 days) level of bioaccessibility for each treatment. This suggests that the stirring and addition of water might have enhanced those highly hydrophobic PAHs bioaccessibility for a short time before PAHs sorbed to soil particles, either because they could or were not yet dissipated.

(iv) The global residual and bioaccessible contents of all PAHs confirm some previously made observations. The total residual PAHs content is significantly lower ($p<0.05$) after 14 days for E_TP samples and after 28 days for C samples but is not different after 28 days for E_MS samples. On the other hand the total bioaccessible PAHs content is highly significantly lower ($p=0.000$) than prior the incubation after 14 days for E_MS samples and after 28 days for C samples. Here again this suggests that TP exudates enhanced soil microbial activity, allowing PAHs dissipation to start faster than in C samples. This hypothesis is supported by the significantly more important CO_2 emissions observed in E_TP samples (Figure 2) and the higher dehydrogenase activity (showing soil microbiota enhanced activity) in Figure 3. But this easily available carbon source was also probably favoured to PAHs throughout the incubation (Cébron et al. 2011), which could explain why C and E_TP total residual contents are statistically not different after 28 days of incubation. As for MS exudates negatively influencing PAHs dissipation, it is reinforced by the fact that CO_2 emissions in E_MS samples were not different from the ones in C samples, suggesting that MS exudates were not favoured to PAHs

as a carbon source but also that there was not much mineralization taking place in the microcosm. Such results are surprising since MS exudates should also constitute an easily accessible source of carbon for the microbiota, and dehydrogenase activities were also enhanced in the presence of MS exudates.

4. CONCLUSIONS AND PERSPECTIVES

The objectives of the exposed experiments were to adapt a common and cost-effective Tenax ® beads extraction protocol to an aged-contaminated soil that would serve as a comparison basis for PAHs bioaccessibility measurements; and to evaluate the role of *Medicago sativa* L. and *Trifolium pratense* L. root exudates in enhancing PAHs bioaccessibility and biodegradation in an aged-contaminated soil.

PAHs desorption kinetics were established and described by the site distribution model. The models' parameters helped calculate minimal extraction times for all compounds and a common extraction time was determined (48 h).

The results from the incubation experiment strongly suggest that the global dissipation of PAHs is not enhanced by the presence of *Medicago sativa* L. nor *Trifolium pratense* L. root exudates at least in a relatively short time (28 days) and is equivalent in control samples.

This suggests that humidification, oxygenation and a little heating is enough for the natural microbiota to attenuate the pollution, rendering other treatments pointless. However, the parallel diminution of PAHs bioaccessibility and dehydrogenase activity suggest that dissipation in untreated samples is likely to reach a limit. Indeed in a logic of soil remediation through biodegradation (for which the dissipation must be carried as far as possible and the microbiota must reach the contaminants), the balance between mass transfer and microbial degradation should be maintained (Johnsen et al. 2005). In order to achieve that, bioaccessible contents would have to remain similar until the dissipation is more advanced, and it is not the case here. On the other hand, a diminution of the bioaccessible contents also means the threat to the environment is diminished because the remaining contaminants are more strongly sorbed to soil particles and thus less likely to be accessed by soil organisms through the soil's aqueous phase, which is positive from a risk analysis point of view.

The incubation period was a norm-based protocol decision and a longer incubation might have shown different results on the long-term. The increase of dehydrogenase activities in presence of both *Medicago sativa* L. and *Trifolium pratense* L. root exudates show a temporary improvement of soil microbial activity. Therefore, a longer pre-incubation period followed by regular exudates inputs might have allowed the dissipation of bioaccessible PAHs before exudates were added. Maybe such treatment would, in the presence of *Trifolium pratense* L. root exudates, improve soil microbial activity on the long term or eventually influence PAHs bioaccessibility. This would be coherent with the hypothesis that *Trifolium pratense* amendments were preferably used as a carbon source by the soil microbiota throughout the incubation. However it does not explain why PAHs bioaccessibility is globally lowered in presence of *Medicago sativa* exudates whilst the global content is not.

Medicago sativa L. and *Trifolium pratense* L. are both *Fabaceae* species, possess a fibrous root system and are nitrogen-independent due to symbiotic relationships with nitrogen fixating rhizobia (Hall et al. 2011). The similarities would be expected to extend to their root exudates characteristics but evidently differences led to different outcomes on PAHs bioaccessibility and dissipation in soil.

The experiment was initially designed based on the knowledge that *Fabaceae* root exudates produce surface-active compounds and under the hypothesis that they could enhance organic compounds bioaccessibility. However, studies on surfactants also mention that hydrophobic

interactions can take place between surfactants and soil particles (Laha et al. 2009), and that partitioning of HOCs into soil-sorbed surfactants could enhance the contaminants sorption to soil. Similar assumptions were made in a previous study aiming to increase PAHs apparent solubility in presence of saponins from *Quillaja saponaria* bark (Davin et al. 2018). The results showed that if the surfactant concentration was too elevated, PAHs solubilisation was less efficient, maybe because PAHs were secluded by saponins micelles or hemimicelles.

The reasons for a diminution of global PAHs bioaccessibility in presence of *Medicago sativa* L. root exudates would have to be investigated through the extraction, characterization and testing of surface-active compounds in exudates (many protocols relying on chromatographic and spectral techniques exist and have been reviewed by Oleszek and Bialy (2006). If *Medicago sativa* L. exudates turned out to present stabilization properties towards organic contaminants such as PAHs, maybe this type of amendment could be investigated as a secluding agent to slow down a pollution migration, for example.

For now and from a PAHs-remediation point of view, the results suggest that *Medicago sativa* L. and *Trifolium pratense* L. root exudates, when added in a single dose, do not enhance PAHs bioaccessibility in the tested soil, and that simple soil moisturizing and incubation, as applied in control samples, leads to identical PAHs dissipation, at least on the short-term. However, it would be of great interest to evaluate whether the growth of whole *Medicago sativa* L. or *Trifolium pratense* L. plants on contaminated soils affects PAHs bioaccessibility and dissipation in similar ways, given that root exudates are released at different, continuous rates in situ.

Reference list

AFNOR XP U44-163 Amendements organiques et supports de culture - Caractérisation de la matière organique par la minéralisation potentielle du carbone et de l'azote.

Alagić S, Maluckov BS, & Radojičić VB 2015. How can plants manage polycyclic aromatic hydrocarbons? May these effects represent a useful tool for an effective soil remediation? A review. *Clean Technol. Environ. Policy* 17(3), 597–614.

Alegbeleye OO, Opeolu BO, & Jackson VA 2017. Polycyclic Aromatic Hydrocarbons: A Critical Review of Environmental Occurrence and Bioremediation. *Environ. Manage.* 60(4), 758–783.

Alves WS, Manoel EA, Santos NS, Nunes RO, Domiciano GS, & Soares MR 2018. Phytoremediation of polycyclic aromatic hydrocarbons (PAH) by cv. Crioula: A Brazilian alfalfa cultivar. *Int. J. Phytoremediation* 20(8) 747-755.

Barnier C, Ouvrard S, Robin C, Morel JL. 2014. Desorption kinetics of PAHs from aged industrial soils for availability assessment. *Sci Total Environ.* 470–471:639–645.

Cébron A, Louvel B, Faure P, France-lanord C, Chen Y, Murrell JC, Leyval C. 2011. Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. *Environ. Microbiol*. 13(3):722–736.

Connaughton DF, Stedinger JR, Lion L.W., & Shuler M.L. 1993. Description of time-varying desorption kinetics: release of naphthalene from contaminated soils. *Environ. Sci. Technol.* 27(12) 2397–403.

Cornelissen G, Van Noort PCM, Govers HAJ. 1997. Desorption kinetics of chlorobenzenes, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls: Sediment extraction with Tenax® and effects of contact time and solute hydrophobicity. *Environ Toxicol Chem.* 16(7):1351–1357.

Das S., Varma A. 2011. Roles of enzymes in maintaining soil health. In: Shukla, G., Varma, A., eds, *Soil Enzymology*, 1st ed, Vol 22 – Soil Biology. Springer-Verlag, Berlin, Heidelberg, Germany, pp. 25-42.

Davin M, Starren A, Deleu M, Lognay G, Colinet G, Fauconnier M-L. 2018. Could saponins be used to enhance bioremediation of polycyclic aromatic hydrocarbons in aged-contaminated soils? *Chemosphere.* 194:414–421.

Ehlers LJ, Luthy RG. 2003. Contaminant bioavailability in improving risk assessment and remediation rests on better understanding bioavailability. *Environ Sci Technol.* 37:295–302.

Hall J, Soole K, Bentham R. 2011. Hydrocarbon phytoremediation in the family Fabaceae-a review. *Int. J. Phytoremediation.* 13(4):317–332.

Hamdi H, Benzarti S, Aoyama I, Jedidi N. 2012. Rehabilitation of degraded soils containing aged PAHs based on phytoremediation with alfalfa (*Medicago sativa* L.). *Int. Biodeterior. Biodegrad.* 67:40-47.

Haritash AK, Kaushik CP. 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater.* 169(1–3):1–15.

Hoagland DR. & Arnon DI. 1950. *The water-culture method for growing plants without soil.* California Agricultural Experiment Station, Circular-347.

Institut National de l'Environnement Industriel et des Risques (INERIS) 2005. Hydrocarbures Aromatiques Polycyliques - Guide méthodologique - Acquisition des données d'entrée des modèles analytiques ou numériques de transferts dans les sols et les eaux souterraines. 53(9):1689–1699. doi:10.1017/CBO9781107415324.004.

ISO 11465:1993 cor 1994 - Soil quality - Determination of dry matter and water content on a mass basis - Gravimetric method.

ISO 13877:1998 Soil quality - Determination of polynuclear aromatic hydrocarbons - Method using high -performance liquid chromatography.

Johnsen AR, Wick LY, Harms H. 2005. Principles of microbial PAH-degradation in soil. *Environ Pollut.* 133:71–84.

Kass RE, Raftery AE. 1995. Bayes Factors. *J. Am. Stat. Assoc.* 90(430):773–795.

Kobayashi T, Kaminaga H, Navarro RR, Iimura Y. 2012. Application of aqueous saponin on the remediation of polycyclic aromatic hydrocarbons-contaminated soil. *J. Environ. Sci. Health. - Part A Tox/Hazard Subst. Environ. Eng.* 47:1138–1145.

Kregiel D, Berlowska J, Witonska I, Antolak H, Proestos C, Babic M, Babic L, Zhang B. 2017. Saponin-Based, Biological-Active Surfactants from Plants. In: Najjar R., ed, *Application and Characterization of Surfactants*, IntechOpen. DOI: 10.5772/65591

Laha S, Tansel B, Ussawarujikulchai A. 2009. Surfactant-soil interactions during surfactant-amended remediation of contaminated soils by hydrophobic organic compounds: A review. *J Environ. Manage.* 90:95–100.Von Lau E, Gan S, Ng HK, Poh PE. 2014. Extraction agents for the removal of polycyclic aromatic hydrocarbons (PAHs) from soil in soil washing technologies. *Environ Pollut.* 184:640–649.

Louvel B. 2010. Etude en microcosmes de l'effet du ray-gras et de ses exsudats racinaire sur la dissipation des HAP et les communautés bactériennes dégradantes. Université de Lorraine, Cours Léopold, Nancy, France.

Martin BC, George SJ, Price CA, Ryan MH, & Tibbett M 2014. The role of root exuded low molecular weight organic anions in facilitating petroleum hydrocarbon degradation: Current knowledge and future directions. *Sci Total Environ.* 472: 642–653.

Oleszek W, Bialy Z. 2006. Chromatographic determination of plant saponins-An update (2002-2005). *J. Chromatogr. A.* 1112:78–91.

Ouvrard S, Chenot ED, Masfaraud JF, Schwartz C. 2013. Long-term assessment of natural attenuation: Statistical approach on soils with aged PAH contamination. *Biodegradation*. 24(4):539-548.

Ouvrard S, Leglize P, & Morel JL 2014. PAH Phytoremediation: Rhizodegradation or Rhizoattenuation? *Int. J. Phytoremediation* 16(1), 46–61.

Prague M, Diakite A, Commenges D. 2012. Package 'marqLevAlg' - Algorithme de Levenberg-Marquardt en R : Une alternative à 'optimx' pour des problèmes de minimisation. 1ères Rencontres R, Bordeaux, France. <hal-00717566>

Richardson SD, Aitken MD. 2011. Desorption and bioavailability of polycyclic aromatic hydrocarbons in contaminated soil subjected to long-term in situ biostimulation. *Environ Toxicol. Chem.* 30(12):2674–2681.

Semple KT, Doick KJ, Jones KC, Burauel P, Craven A, Harms H. 2004. Peer Reviewed: Defining Bioavailability and Bioaccessibility of Contaminated Soil and Sediment is Complicated. *Environ. Sci. Technol.* 38(12):228A-231A.

Semple KT, Morriss AWJ, Paton GI. 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *Eur. J. Soil. Sci.* 54:809–818.

Shaw L, Burns R. 2005. Soil microbial activity. In: Bloem J, Hopkins DW, Benedetti A, eds., *Microbiological methods for assessing soil quality.* CABI Publishing, Cambridge, MA, USA.

Uroz S, Courty PE, & Oger P 2019. Plant symbionts are engineers of the plant-associates microbiome. *Trends Plant Sci.* 24(10) 905-916.

Vincken JP, Heng L, de Groot A, Gruppen H. 2007. Saponins, classification and occurrence in the plant kingdom. *Phytochemistry.* 68:275–297.

Wei S, & Pan S 2010. Phytoremediation for soils contaminated by phenanthrene and pyrene with multiple plant species. *J. Soils Sediments* 10, 886-894.

Yu L, Duan L, Naidu R, & Semple KT. 2018. Abiotic factors controlling bioavailability and bioaccessibility of polycyclic aromatic hydrocarbons in soil: Putting together a bigger picture. *Sci. Total Environ*, 613–614, 1140–1153.

Figures and Tables

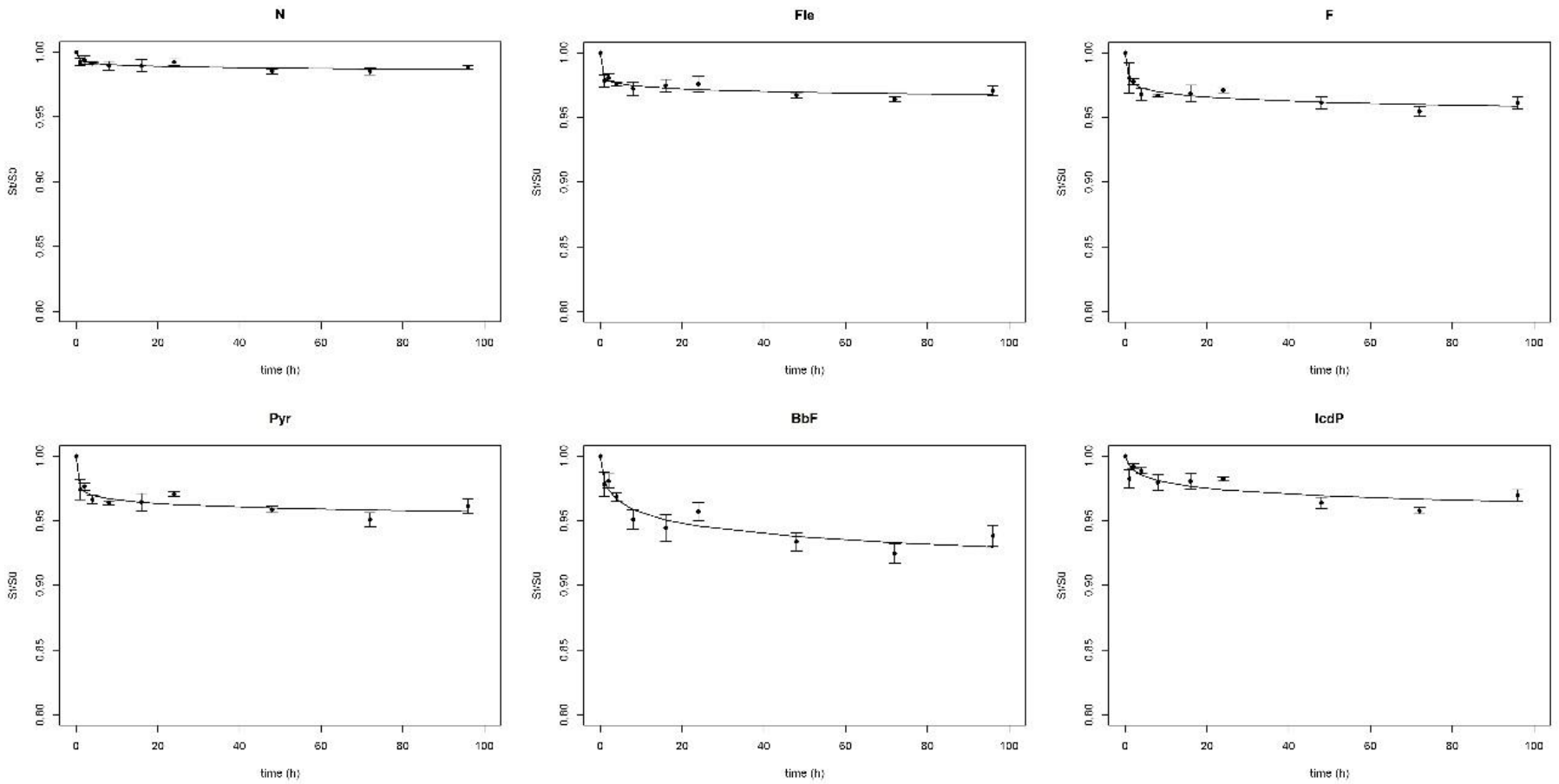
Table 1. Desorption theoretical models and their characteristics.

Desorption model	Equation	Number of parameters
First-order model	$\frac{S_t}{S_0} = e^{-kt}$	1
First-order two-compartment model	$\frac{S_t}{S_0} = F_{rap} * e^{-k_{rap}t} + F_{slow} * e^{-k_{slow}t}$ $F_{rap} + F_{slow} = 1$	4
First-order three-compartment model	$\frac{S_t}{S_0} = F_{rap} * e^{-k_{rap}t} + F_{int} * e^{-k_{int}t} + F_{slow} * e^{-k_{slow}t}$ $F_{rap} + F_{int} + F_{slow} = 1$	6
Site distribution model	$\frac{S_t}{S_0} = \left(\frac{\beta}{\beta + t}\right)^{\alpha}$	2

Table 2. Fitted parameters of the site distribution model for the different PAHs and t_{ex} values calculated according to equation (3).

PAHs*	β (h)	α (-)	t_{ex} (h)
N	$1.54.10^{-2}$	$1.53.10^{-3}$	24
Ace	$6.82.10^{-4}$	$1.22.10^{-3}$	24
Fle	$8.98.10^{-4}$	$2.83.10^{-3}$	24
Phen	$2.00.10^{-3}$	$3.91.10^{-3}$	48
Anthr	$9.30.10^{-3}$	$1.27.10^{-2}$	48
F	$1.05.10^{-2}$	$4.61.10^{-3}$	48
Pyr	$2.43.10^{-3}$	$4.14.10^{-3}$	48
BaA	$1.02.10^{-1}$	$1.14.10^{-2}$	48
Chrys	$1.24.10^{-1}$	$1.53.10^{-2}$	48
BbF	$2.78.10^{-1}$	$1.24.10^{-2}$	48
BkF	$6.03.10^{-1}$	$1.45.10^{-2}$	48
BaP	$5.54.10^{-1}$	$1.12.10^{-2}$	48
DBahA	$1.34.10^{0}$	$1.15.10^{-2}$	48
BghiP	$1.95.10^{-1}$	$4.66.10^{-3}$	48
IcdP	$5.29.10^{-1}$	$6.88.10^{-3}$	48

*PAHs are sorted by increasing molecular weight



510

511

512 **Fig. 1 Examples of PAHs desorption kinetics using Tenax®. S_t/S_0 is the remaining sorbed fraction according to extraction time. Dots are data means ± confidence interval (n=5), lines**
513 **are fitted site distribution models**

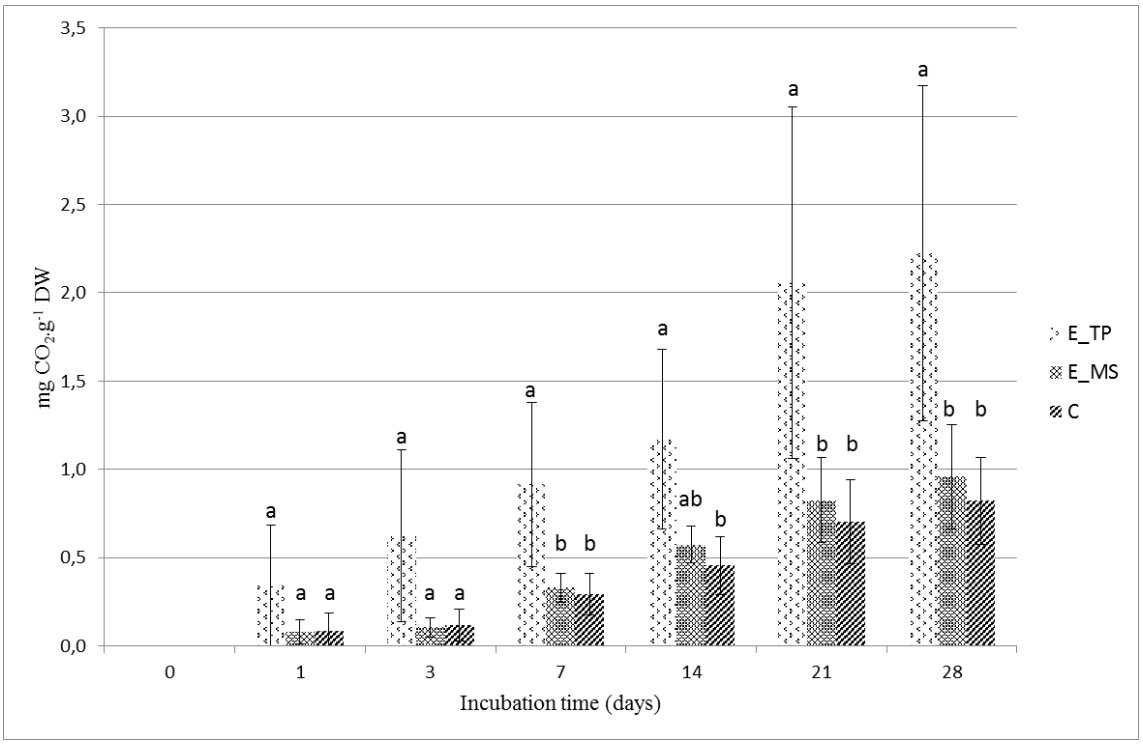


Fig. 2 CO_2 cumulated emissions during the incubation of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates compared to untreated samples (C). Values are means ± confidence interval (α=5%). Within each time group, treatments sharing the same letter are not significantly different (p > 0.05)

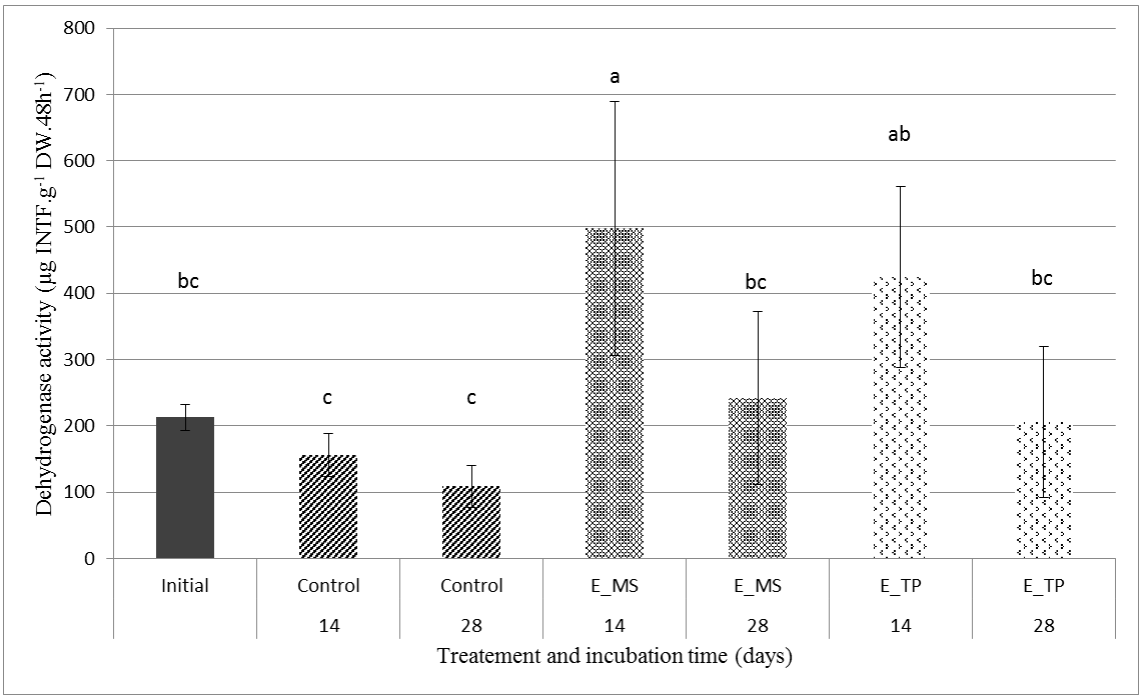


Fig. 3 Dehydrogenase activities of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates, compared to untreated samples (C) after different incubation periods. Values are means ± confidence interval (α=5%). There is a significant interaction between time and treatment. Sticks that share the same letter are not significantly different (p > 0.05)

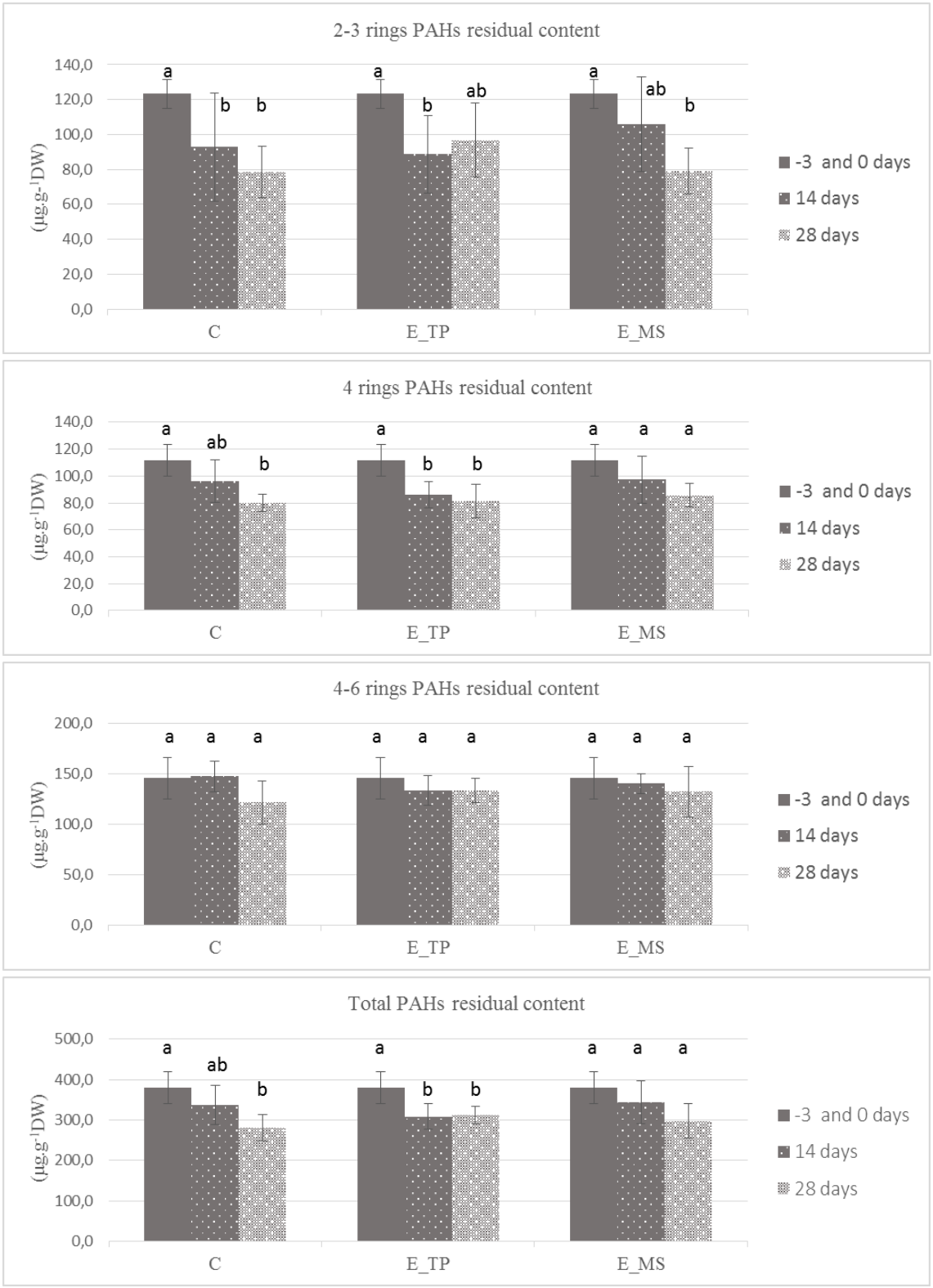


Fig. 4 PAH residual contents of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates, compared to untreated samples (C) after different incubation periods. Values are means ± confidence interval (α=5%). Within each treatment group, sticks that share the same letter are not significantly different (p > 0.05)

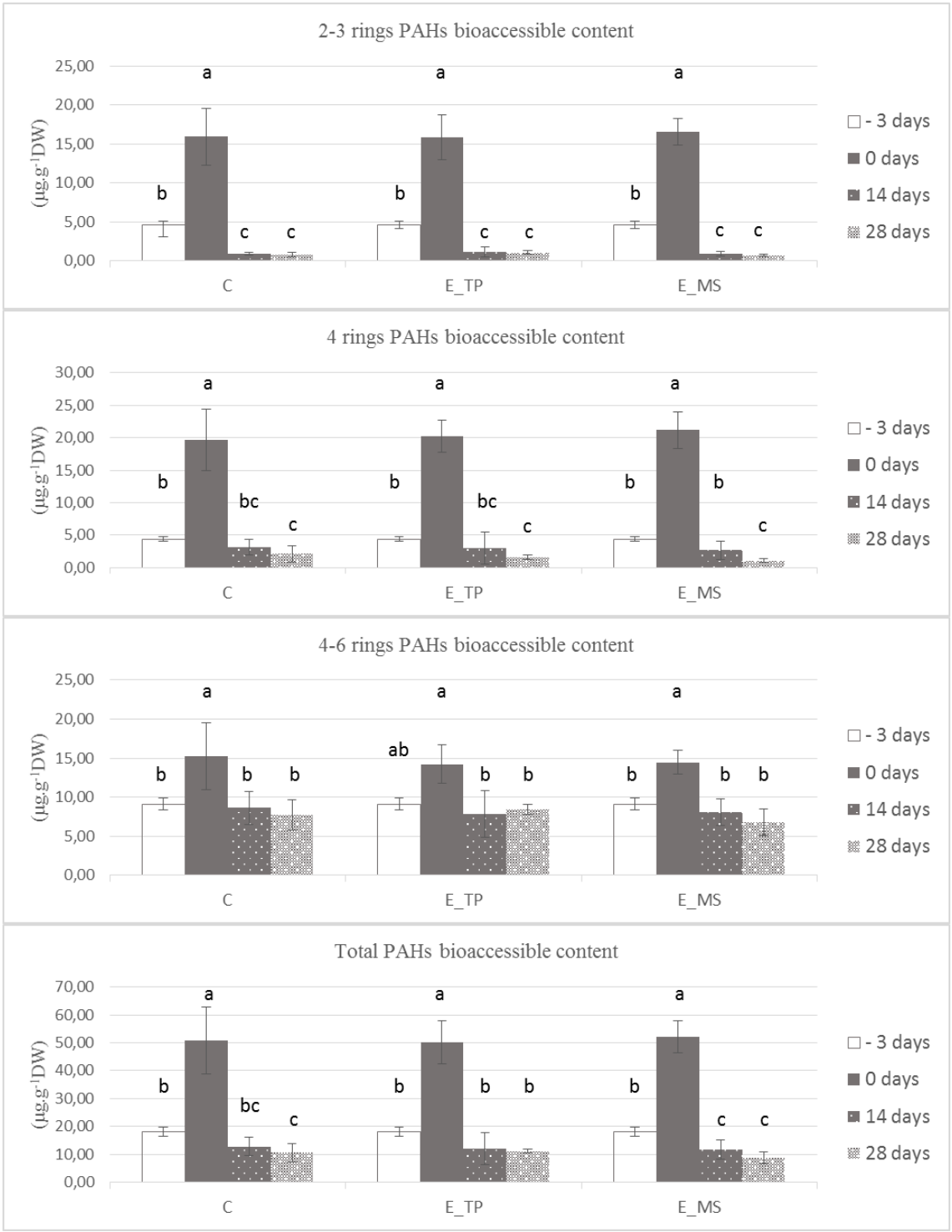


Fig. 5 PAH bioaccessible contents of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates, compared to untreated samples (C) after different incubation periods. Data before and after the pre-incubation period are respectively named "-3 days" and "0 days" Values are means ± confidence interval (α=5%). Within each treatment group, sticks that share the same letter are not significantly different (p > 0.05).

ESM 1 PAHs total initial concentrations and PAHs extracted amounts after different times of extraction by Tenax® beads. Values were used to calculate remaining sorbed fractions for each time of extraction, according to equation (1)

	time (h)	N	Ace	Fle	Phen	Anthr	F	Pyr	BaA
Total concentration ($\mu g.g^{-1}DW$)	0	28.9 ± 1.7	19.4 ± 1.2	12.5 ± 1.1	46.5 ± 5.5	16 ± 1.4	65.9 ± 7.1	45.6 ± 4.8	28.3 ± 3.6
Bioaccessible concentration ($\mu g.g^{-1}DW$)	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	1	0.22 ± 0.09	0.21 ± 0.05	0.27 ± 0.06	1.18 ± 0.27	0.91 ± 0.18	1.28 ± 0.75	1.18 ± 0.35	0.83 ± 0.26
	2	0.18 ± 0.08	0.16 ± 0.03	0.24 ± 0.04	1.04 ± 0.11	1.03 ± 0.20	1.46 ± 0.16	1.07 ± 0.12	0.85 ± 0.18
	4	0.25 ± 0.03	0.20 ± 0.01	0.30 ± 0.02	1.51 ± 0.22	1.21 ± 0.10	2.11 ± 0.29	1.53 ± 0.16	1.21 ± 0.10
	8	0.30 ± 0.09	0.24 ± 0.02	0.35 ± 0.06	1.53 ± 0.07	1.34 ± 0.05	2.17 ± 0.08	1.64 ± 0.07	1.58 ± 0.10
	16	0.30 ± 0.13	0.22 ± 0.06	0.32 ± 0.06	1.58 ± 0.27	1.47 ± 0.32	2.08 ± 0.42	1.62 ± 0.29	1.54 ± 0.32
	24	0.22 ± 0.07	0.19 ± 0.04	0.30 ± 0.07	1.56 ± 0.53	1.44 ± 0.35	1.90 ± 0.12	1.33 ± 0.08	1.36 ± 0.13
	48	0.42 ± 0.05	0.29 ± 0.04	0.41 ± 0.03	1.79 ± 0.11	1.70 ± 0.44	2.54 ± 0.29	1.88 ± 0.11	2.00 ± 0.21
	72	0.43 ± 0.08	0.34 ± 0.08	0.45 ± 0.02	2.04 ± 0.17	1.99 ± 0.27	2.97 ± 0.24	2.24 ± 0.24	2.35 ± 0.20
	96	0.34 ± 0.04	0.25 ± 0.07	0.37 ± 0.05	1.81 ± 0.20	1.51 ± 0.23	2.55 ± 0.31	1.77 ± 0.25	1.98 ± 0.24

	time (h)	Chrys	BbF	BkF	BaP	DBahA	BghiP	IcdP
Total concentration ($\mu g.g^{-1}DW$)	0	32.4 ± 4.0	23.1 ± 3.3	11.8 ± 1.6	18.3 ± 2.6	2.9 ± 0.1	14.1 ± 3.6	15 ± 2.6
Bioaccessible concentration ($\mu g.g^{-1}DW$)	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	1	1.12 ± 0.38	0.50 ± 0.21	0.24 ± 0.08	0.33 ± 0.10	0.06 ± 0.03	0.22 ± 0.08	0.26 ± 0.10
	2	1.26 ± 0.40	0.44 ± 0.12	0.20 ± 0.06	0.23 ± 0.05	0.04 ± 0.00	0.12 ± 0.02	0.12 ± 0.03
	4	1.75 ± 0.12	0.73 ± 0.08	0.33 ± 0.03	0.40 ± 0.03	0.04 ± 0.01	0.16 ± 0.03	0.17 ± 0.03
	8	2.27 ± 0.15	1.13 ± 0.17	0.55 ± 0.06	0.66 ± 0.05	0.07 ± 0.01	0.25 ± 0.07	0.30 ± 0.09
	16	2.25 ± 0.56	1.28 ± 0.23	0.51 ± 0.13	0.62 ± 0.18	0.06 ± 0.02	0.23 ± 0.06	0.29 ± 0.09
	24	2.06 ± 0.41	0.99 ± 0.15	0.46 ± 0.05	0.57 ± 0.06	0.06 ± 0.01	0.24 ± 0.03	0.26 ± 0.02
	48	2.77 ± 0.34	1.52 ± 0.16	0.76 ± 0.03	0.95 ± 0.07	0.12 ± 0.03	0.43 ± 0.04	0.54 ± 0.06
	72	3.42 ± 0.24	1.73 ± 0.17	0.9 ± 0.07	1.14 ± 0.06	0.17 ± 0.06	0.51 ± 0.05	0.63 ± 0.04
	96	2.98 ± 0.27	1.42 ± 0.17	0.8 ± 0.11	0.95 ± 0.11	0.13 ± 0.02	0.32 ± 0.09	0.46 ± 0.06

543 **ESM 2 BIC values calculated for each desorption model of each PAH, according to equation (2)**

	Model			
PAHs	1 order	1 order - 2 compartment	1 order - 3 compartment	Site distribution
N	-422*	-411	-401	-352
Ace	-428*	-411	-396	-337
Fle	-385*	-368	-372	-259
Phen	-362*	-313	-350	-237
Anthr	-249*	-242	-237	-141
F	-356*	-343	-351	-244
Pyr	-356*	-341	-346	-234
BaA	-313*	-302	-303	-202
Chrys	-285*	-276	-277	-179
BbF	-310*	-303	-301	-214
BkF	-318*	-312	-310	-232
BaP	-336*	-334	-317	-253
DBahA	-286	-277	-291*	-265
BghiP	-352	-349	-356*	-297
IcdP	-340	-331	-345*	-291

544