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Implementing standardized desorption extraction into bioavailability-oriented bioremediation of PAH-polluted soils

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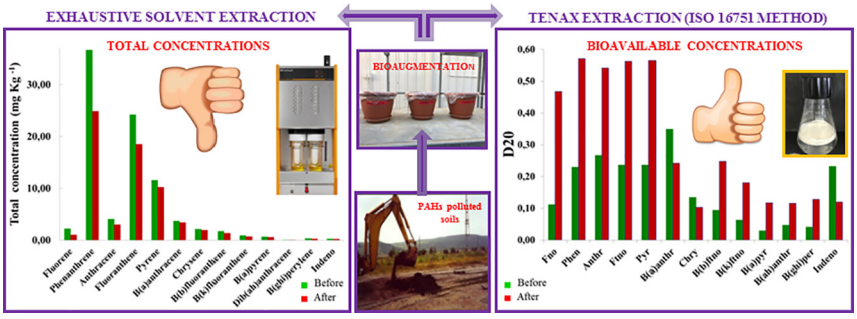
HIGHLIGHTS

- Standardized desorption was used to measure environmental bioavailability of PAHs.
- Bioavailable concentrations differ significantly from total concentrations.
- The method is applicable to a wide range of soils and bioremediation approaches.
- Traditional bioremediation is compared with bioavailability-oriented bioremediation.

GRAPHICAL ABSTRACT



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ABSTRACT

We applied a standardized desorption extraction method (Tenax extraction), to assess the bioavailability of native polycyclic aromatic hydrocarbons (PAHs) present in contaminated soils. Single-time point Tenax extraction at 20 h has been recently proposed by the International Organization for Standardization as one of the chemical methods to measure environmental bioavailability of nonionic pollutants (ISO/TS 16751). This work is one of the first ones that use this ISO method systematically in the field of bioremediation, and shows its advantages when used in combination with total concentrations determined with conventional, exhaustive solvent extraction. This method has been applied to different PAHs contaminated soils which had a different level of total PAHs (66–4370 mg kg^{-1}) and which were from different contaminated sites and dissimilar bioremediation approaches. In most samples the study was focused on phenanthrene and benzo(a)pyrene as representative pollutants, although the profile of total PAHs was also studied in some samples. The results from this study show that the pollutant fractions extracted with Tenax during 20 h (D20) decreased after traditional bioremediation (biostimulation and phytoremediation), but they often increased in bioavailability-oriented treatments involving either biosurfactants or bioaugmentation with specialized microbial inocula. Therefore, D20-based assessments provided information on the bioremediation performance, not directly evident through the measurement of total PAH concentrations.

1. Introduction

The bioavailability of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), pesticides, biocides and pharmaceuticals in soils and sediments is an important area of scientific research in

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environmental sciences. Bioavailability has a great consideration in the study of bioaccumulation and toxicity in soils and sediments. However, implementation of bioavailability in environmental regulatory frameworks remains difficult because scientific developments on bioavailability are not always translated into ready-to-use approaches for regulators and, therefore, no integrated approach for implementation is available. Traditionally, only the total-extractable concentrations are considered in the assessments of toxicity and exposure of organic compounds in soils and sediments. Recently, a group of authors from academia, industry and regulation discussed bioavailability concepts and methods (Ortega-Calvo et al., 2015), and proposed a simplified approach in which the assessments of contamination in soils and sediments should be based on two measurable values: the total extractable concentration and the bioavailable concentration. In this context, a very important question is how should bioavailability be measured. Several experimental methods have been developed to assess environmental bioavailability of hydrophobic organic contaminants (HOCs), in the context of ecological health risk assessments. One of the chemical methods which has been proposed is the desorption extraction with Tenax during 20 h, and it has recently been included in the only standardized bioavailability methodology (ISO method 16751), known so far (ISO/TS 16751, 2018; Ortega-Calvo et al., 2015; Cornelissen et al., 2001; Lydy et al., 2015). Using Tenax-extractable concentrations in soils and sediments risk assessments has several advantages: is rapid, simple, straightforward, and relatively inexpensive (Harwood et al., 2015). In spite of these advances, further efforts are needed to demonstrate the wide applicability of this methodology to a variety of HOCs and environmental matrices, for these approaches to finally settle down in environmental regulations.

A relevant aspect of the bioavailability of HOCs connects with their environmental persistence, especially when methods based on the biodegradation by microbial communities are employed for remediation of soils and sediments. There are many techniques and strategies of bioremediation, as for example they include natural attenuation, bioaugmentation, biostimulation and phytoremediation. The effectiveness of these methods in different soils depends on several factors (environmental conditions, oxygen availability, pH, temperature, *etc.*) but one of the most important ones is a limited bioavailability (Bamforth and Singleton, 2005; Harmsen and Rietra, 2018). When traditional bioremediation approaches fail to achieve further decreases in pollutant concentrations due to these factors, it is possible to operate on pollutant-phase exchange mechanisms and microbial dispersal to enhance bioavailability and biodegradation (Maliszewska-Kordybach and Smereczak, 2000; Tejeda-Agredano et al., 2013; Bueno-Montes et al., 2011). In this context, bioavailability measurements with standardized methods represent an added value for bioremediation strategies, as they can systematically be used by industries and regulators to evaluate more realistically remediation operations and end-points, in addition to assessments based on total pollutant concentrations only.

The present study investigated the effect of bioremediation on bioavailability, determined as the fraction of PAHs extracted with Tenax during 20 h (D20) in a set of 11 samples which were selected for their different level and origin of pollution, as well as bioremediation approaches used. In most samples the study was focused on phenanthrene and benzo(a)pyrene as representative pollutants for, respectively, directly mineralizable and cometabolizable chemicals, although the profile of total PAHs was also studied in some samples. The objectives of this study were to a) study the effect of traditional bioremediation on D20, and b) study the effect of bioavailability-oriented bioremediation on D20. As a quality assurance, we also characterized the desorption kinetics of the full profile of PAHs in a representative, untreated soil sample, to highlight the importance of ensuring the 20 h time window to capture the fast desorption when using this standardized method, for a specific group of chemicals, like PAHs.

2. Materials and methods

2.1. Chemicals

Analytical grade dichloromethane, acetonitrile, hexane and acetone were supplied by Fischer Chemical (Canada). Tenax (60–80 mesh) 177–250 μm was supplied by Buchem BV (Netherlands). The internal standard 1-fluoropyrene (0.1 mg mL^{-1} in toluene) was purchased from Chiron AS (Norway). Rhamnolipid biosurfactant (R90, 90% pure) was supplied by AGAE Technologies (Oregon, USA).

2.2. Soils

The set of soils used in this study were PAH-contaminated soils, selected according to their different levels of contamination and to the dissimilar bioremediation treatments they received (Table 1). These soils exhibited three different types of industrial pollution: a) creosote pollution from a wood-treating facility in Andujar (Spain), b) pollution by PAHs, BTEX and alkanes from an industrial exploitation in Fidenza (Italy), and c) pollution by subproducts from a metallurgic plant in Normandy (France). The samples 1, 3, 6, and 8, and 10 were original untreated samples, whereas the rest was the result of the bioremediation treatments described in Table 1. In our approach, we considered, as traditional bioremediation treatments, those well-established methods involving biostimulation with inorganic nutrients and phytoremediation. As bioavailability-oriented treatments, we considered biostimulation with biosurfactants, after a first cycle of traditional bioremediation, and bioaugmentation with a consortium composed by specialized microbial strains. Once obtained, all samples used in this study were air-dried, sieved (2 mm mesh) and homogenized.

All soil samples contaminated by creosote that underwent bioremediation were obtained by combining the original soil (which was highly contaminated by PAHs) with an uncontaminated soil (which was sterilized previously) in order to obtain a soil with specific PAH concentration and texture. The mixture of both soils was done in a tumble mixer. The relation contaminated/uncontaminated soil depended on the specific objective in each experiment. In this way, to obtain the untreated sample 1, the uncontaminated soil was from the agricultural experimental station of the University of Barcelona and in this case a total of 20 kg of the agricultural soil was combined with 10 kg of soil from the creosote site (2:1 w/w) and the mixture was homogenized in a tumbler mixer for 24 h with regular changes in the direction of rotation. Sample 2 was a biostimulated soil originated from sample 1, which had been amended with urea and K_2HPO_4 to reach a C:N:P ratio of 300:10:1 and then treated in dynamic biopiles during 5 months, maintaining the water content at 40% of its water holding capacity. The untreated sample 3 was obtained in a similar way as sample 1, but in this case the uncontaminated soil was from the agricultural experimental station from IRNAS (La Hampa). In addition, sand was added to this mixture (the proportion uncontaminated soil and sand was 67:33 w/w). Sample 4 originated from sample 3, and it was obtained after 60 days of phytoremediation, which was carried out in a greenhouse with planted sunflower (*Helianthus annuus*, L). Sample 5 originated from sample 4 after an additional period of 30 days, from the addition of a rhamnolipid biosurfactant (R90) at 7 mg g^{-1} (w/w). All these treatments were performed in triplicate.

The untreated sample 6 was obtained in a similar way as sample 3 but in this case the sand was not used and the proportion of contaminated and uncontaminated soil was 1:100 w/w. Sample 7 originated from sample 6, and it was obtained after 60 days of bioaugmentation. The soil, a microbial consortium and rice husk (which was used as an inoculum carrier, 0.05 kg of rise husk per 40 kg of soil) were homogenized in a cement mixer. The consortium included the following microorganisms: *Fusarium solani*, *Talaromyces sayulitensis*, *Aspergillus jensenii*, *Aspergillus terreus*, *Trametes gibbosa*, *Pseudomonas* spp., *Pseudomonas putida*, *Acinetobacter calcoaceticus* and *Pseudomonas plecoglossicida*.

Table 1
Description of samples used in this study.

Site description	Treatment	Sample number	
		Initial	Final
Creosote soil from a wood-treating facility with a pollution by creosote going back 100 years in Andujar, Jaen, Spain	Biostimulation in 2.5 kg biopiles, using urea and K_2HPO_4, 5 months	1	2
	Phytoremediation with sunflowers, in a greenhouse, 4 kg soil in every pot, 60 days	3	4
	Biostimulation with a biosurfactant, after phytoremediation, in a greenhouse, 30 days	4	5
	Bioaugmentation, 4 kg soil in every pot, 60 days	6	7
Highly contaminated soil by PAHs, BTEX and alkanes from a site with a long history of industrial exploitation, Fidenza, Italy	Bioaugmentation, 4 kg soil in every tray, 60 days	8	9
Contaminated soil that was collected from a highly polluted area situated in a metallurgic plant in Calvados-Colombelles, Normandy, France	Bioaugmentation, 4 kg soil in every pot, 60 days	10	11

The microbial consortia used in the bioaugmentation experiments are microorganisms isolated in the first three meters of depth from the contaminated site in Fidenza, Italy. This consortium was selected because it is composed of the fungal and bacterial consortia which gave the best results in preliminary microcosms in relation to pollutant degrading abilities. This consortium was kindly provided by Fabrizio Beltrametti (Actygea SRL). The total bacterial count at the beginning of this experiment was 10^5 CFU g^{-1} soil. Moreover, the level of nutrients in the soil 7 was corrected with ammonium nitrate (NH_4NO_3) to obtain a C:N:P ratio of 100:15:1.

All bioaugmentation experiments were carried out in the same way. There were three replicates (pots) for every treatment, including a control treatment (without consortia), each containing 4 kg of soil. Three subsamples were collected from each pot, that were mixed to obtain one complex sample per pot. The results have been expressed as an average of these three replicates.

The untreated soil sample 8 was collected from the area of "Ex-Carbochimica", a national interest site in Fidenza (Emilia Romagna, Italy). This site has a long history of industrial exploitation. In 1988, this site run a tar distillery, and the production of lead tetraethyl, sulfuric acid and phosphate fertilizers. In 1945, the site was bombarded during the Second World War. The disposal of industrial installations and contaminated soil remediation using traditional techniques were started by Fidenza Municipality in 2003. This soil was contaminated by PAHs, benzene and alkanes. Heavy metals were strictly confined in a small area already under treatment. Pollutants can be found at high depth (>26 m), including the groundwater. Experimental activities started in July 2016 when the life-biorest project started. More information about this contaminated site can be found elsewhere (Re, 2016). Sample 9 originated from sample 8 after 60 days of bioaugmentation in the same way as sample 7, and the consortium used was similar, including: *Pseudomonas putida*, *Acinetobacter calcoaceticus*, *Cupriavidus* sp. and *Sphingobacterium* sp. The fungi used in the consortia were: *Bjerkandera adusta*, *Aspergillus jensenii*, *Aspergillus terreus*, *Cladosporium cladosporioides* and *Trichoderma harzianum*. This consortium was designed to achieve the biodegradation of the specific contaminants present in the soil. The untreated sample 10 was a soil that was collected from a potential highly polluted area situated in a metallurgic plant in Calvados-Colombelles (Normandy, France). The plant started to operate in 1920 and was bombarded between 1940 and 1944. The wastes generated from this industrial activity were: demineralized water for boilers (sodium hydroxide solution and clorhidric acid), carbon, oil, and greases. The study site was located in the main central which is the unit responsible for the recovery of so-called fatal gases produced in the coking plant and blast furnaces. It is also responsible for the distribution, energy management and management of all plant water. Sample 11 originated from sample 10 and it was obtained after 60 days of bioaugmentation in the same way as sample 7, and the consortium used was the same.

2.3. Exhaustive extraction and analysis of PAHs

To measure the content of native PAHs in the soils, the soil samples (1 g) were mixed and ground with 1 g of anhydrous sodium sulfate and then extracted in a Soxhlet apparatus for 8 h with 100 mL of 1:1 (v/v) dichloromethane/acetone. The extract volume was reduced with a rotary evaporator and it was then cleaned with a Sep-pak Fluorisil cartridge. The cleaned extract was brought to near dryness under a gentle stream of nitrogen. The residue was then dissolved in acetonitrile and filtered through a syringe filter of nylon. PAH analysis was carried out using a Waters HPLC system (Water 2475 Multiλ fluorescence detector and Water 996 photodiode array detector, Water PAH column C18, 5 μm particle size and 4.6 × 250 mm and 1 mL min^{-1} of flow). The mobile phase used was an acetonitrile/milli-Q water gradient. The column was installed in a thermostatic oven at 30 °C. The concentrations of PAHs are reported as mg kg^{-1} of dry soil. The studied PAHs were analysed with a fluorescence detector and its detection limit was 0.093 mg kg^{-1} for phenanthrene and 0.04 mg kg^{-1} for benzo(a)pyrene. Our method of extraction and analysis of PAHs was verified with a certified reference material (industrial soil, BCR®-524) and our results were in good agreement with the certified values (the certified concentrations are 980 mg kg^{-1} and 8.6 mg kg^{-1} for phenanthrene and benzo(a)pyrene, respectively, and the measured concentrations were 1113.22 ± 145.10 mg kg^{-1} and 8.68 ± 2.30 mg kg^{-1} for phenanthrene and benzo(a)pyrene respectively). In addition, 1-fluropyrene (1 mg kg^{-1} soil) was used as an internal standard to calculate the recovery factor (80–90% for all PAHs).

2.4. Measurement of bioavailable concentration by a desorption extraction method

For a single-point Tenax extraction of the soil, 0.5 g dry soil, 35 mL milli-Q water, 0.2 mL of a biocide (formaldehyde 40%), and 0.7 g Tenax TA beads were placed in 50 mL stainless steel centrifuge tubes equipped with a stainless steel sealing (Heraeus-Sorvall, Madrid) and kept at 24 °C and 120 rpm on a rotary shaker (Bueno-Montes et al., 2011). After 20 h, the tubes were centrifuged for 10 min at 17,212*g*. The floating Tenax beads were completely recovered with a spatula and transferred into a 250 mL screw-capped Erlenmeyer flask containing 100 mL of acetone/hexane (1:1). Next, the internal standard was added in most cases and the extract was kept overnight on a rotary shaker operating at 150 rpm. The extract was evaporated to near dryness, redissolved in acetonitrile, and filtered. PAH analysis was performed by HPLC as described in Section 2.3.

This method used to measure bioavailability considers the fraction of PAH extracted with Tenax at 20 h (D20) that represents the majority of the rapidly desorbing fraction but it should be confirmed in the soils. The value of D20 was calculated by dividing the concentration extracted with Tenax at 20 h by the total concentration in the sample. To study the complete desorption kinetics, the method was the same as described above, but for every sampling time the Tenax was replaced by new Tenax. The desorption data are obtained by the following first-order, two-compartment kinetic model (Cornelissen et al., 1998):

$$S_t/S_0 = F_{fast}\ \exp(-K_{fast}t) + F_{slow}\ \exp(-K_{slow}t) \quad (1)$$

In this equation, S_t and S_o (mg) are the soil-sorbed amounts of PAHs at time t (h) and at the start of the experiment, respectively, F_{fast} and

F_{slow} are the fast- and slow-desorbing fractions, and K_{fast} and K_{slow} (h^{-1}) are the rate constants of fast and slow desorption. The values of the different constants and fractions (F_{fast}, F_{slow}, K_{fast}, and K_{slow}) were obtained by the exponential curve fitting. The ln form of Eq. (1) was subjected to curve fitting. Fits were carried out by minimizing the squares of the differences between experimental and calculated values of ln ($St/S0$) (Solver option from software Microsoft Excel).

3. Results and discussion

3.1. The importance of desorption kinetics to measure bioavailability through D20

The ISO method used to measure bioavailability considers that Tenax extraction during 20 h represents most of the rapid desorption fraction. Although not explicitly mentioned in the standard (ISO/TS 16751, 2018), this time window should be confirmed as sufficient to trap F_{fast} in a given set of environmental samples and pollutants studied. Given the numerous studies on desorption kinetics of PAHs from field contaminated materials on which this ISO method is based, we considered unnecessary – and unpractical – to perform an exhaustive kinetic analysis of all samples included in this study. However, to illustrate this requirement, we selected the sample 8 (Findeza, Italy, Table 1) as a representative soil sample, with an average concentration of total PAHs (141 mg kg^{-1}), and with expected high F_{fast} values, in accordance to its untreated history. The results of the desorption kinetics of PAHs in this sample are shown in Table 2. In this soil the most abundant compounds were phenanthrene and fluoranthene and, with these chemicals, the fast-desorbing fraction was the highest, accounting for >50% of the total compound present.

The kinetics of spontaneous desorption of phenanthrene and benzo(a)pyrene appears in Fig. 1. In this figure it can observed that the model (Eq. (1)) allows a good prediction of spontaneous desorption. Furthermore, the figure shows that, in spite of the very different rate constants for fast desorption in these two compounds (K_{fast}, Table 2), the theoretical recovery of the fast desorbing fraction after 20 h of extraction with Tenax was 99.99% for phenanthrene and 93.5% for benzo(a)pyrene, what indicates that this extraction period was sufficient to assess changes in bioavailability. The full profiles of PAHs for total concentration and for D20 are shown in Fig. 2. In addition, in this figure the same parameters are shown also for sample 9 which is originated after 60 days of bioaugmentation. In this figure, we can observe that the profile of total PAHs and D20 were different before and after bioremediation. The most abundant PAHs before bioremediation were the low molecular weight PAHs, what is typical for a soil that has not been bioremediated (Fig. 2A). The concentration of these PAHs decreased after bioremediation, but this did not happen for the high molecular weight PAHs. However, D20 estimations indicated an increased bioavailability after 60 days of bioaugmentation only, for most compounds (Fig. 2B). This suggests that biodegradation would have progressed further with a subsequent treatment of the bioaugmented soil. The profile of D20 can, therefore, give us additional information about the process performance during a bioremediation approach.

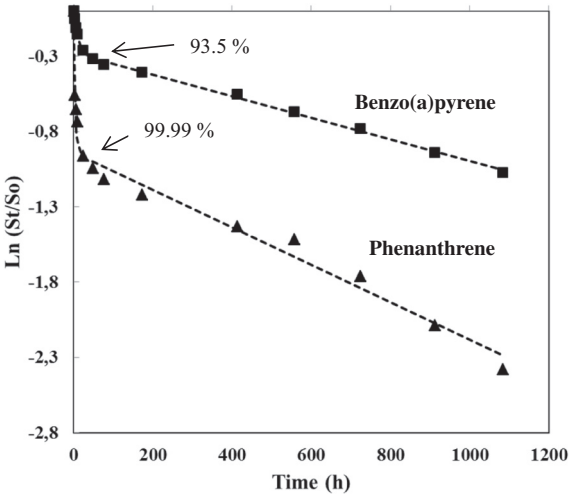

Fig. 1. Kinetics of spontaneous desorption, determined by Tenax extraction, of phenanthrene (triangles) and benzo(a)pyrene (squares) in samples from the area of "Ex-Carbochimica" in Fidenza, Italy (sample 8, Table 1). The dashed lines represent model fitting desorption results to Eq. (1). The percentages in this figure indicate the theoretical recovery of the fast desorbing fraction after 20 h of extraction with Tenax for phenanthrene and benzo(a)pyrene.

3.2. Effect of traditional bioremediation on D20

The following step in this work was to apply single-point desorption extractions to a wide set of samples from different PAH-contaminated soils, differing in PAH content and bioremediation approaches, and to measure the total PAHs concentrations and D20 fractions. We focused on phenanthrene and benzo(a)pyrene as indicator compounds. These results are shown in Table 3. Samples from traditional bioremediation were studied first and, within this group, biostimulation (samples 1 and 2) and phytoremediation (samples 3 and 4). On Table 3, the sample 1 has the highest values of total concentration and D20 for phenanthrene. This result was expected because this sample was not bioremediated and therefore *a priori* enriched in fast-desorbing PAHs. The lower D20 value in the sample 2 for the two compounds, as

Table 2
Kinetic parameters for desorption with Tenax in a soil highly contaminated by PAHs, BTEX and alkanes from Fidenza, Italy (sample 8 in Table 1).

PAH	Concentration (mg kg^{-1})	F_{fast} [a] (%)	K_{fast} [b] (h^{-1})	K_{slow} [c] (10^{-3} h^{-1})
Fluorene	3.14 ± 1.44	42.6 ± 12.0	0.79 ± 0.3	0.59 ± 0.15
Phenanthrene	46.3 ± 9.07	54 ± 18	0.46 ± 0.21	1.4 ± 0.1
Anthracene	2.66 ± 1.07	36 ± 7.9	0.61 ± 0.43	1.5 ± 1.1
Fluoranthene	40.1 ± 8.57	56 ± 5.4	0.26 ± 0.06	0.46 ± 0.24
Pyrene	26.8 ± 4.23	43 ± 3.5	0.37 ± 0.09	0.28 ± 0.12
Benzo(a)anthracene	7.06 ± 1.47	35 ± 7.5	0.20 ± 0.15	0.17 ± 0.04
Crysene	5.10 ± 1.05	44 ± 7.1	0.19 ± 0.11	0.46 ± 0.26
Benzo(b)fluoranthene	4.5 ± 0.87	44 ± 4.3	0.27 ± 0.18	0.8 ± 0.5
Benzo(k)fluoranthene	1.50 ± 0.17	44.7 ± 3.9	0.13 ± 0.055	0.72 ± 0.56
Benzo(a)pyrene	1.37 ± 0.42	28.9 ± 3.3	0.081 ± 0.01	1.7 ± 0.66
Dibenzo(ah)anthracene	0.27 ± 0.06	31.2 ± 7.4	0.048 ± 0.008	0.67 ± 0.035
Benzo(ghi)perylene	0.74 ± 0.17	32.0 ± 11.0	0.20 ± 0.18	2.0 ± 0.99
Indeno(1,2,3-cd)pyrene	1.06 ± 0.48	17.3 ± 0.2	0.053 ± 0.01	0.25 ± 0.043

[a] Fast desorbing fraction.
[b] Rate constant of fast desorption.
[c] Rate constant of slow desorption.

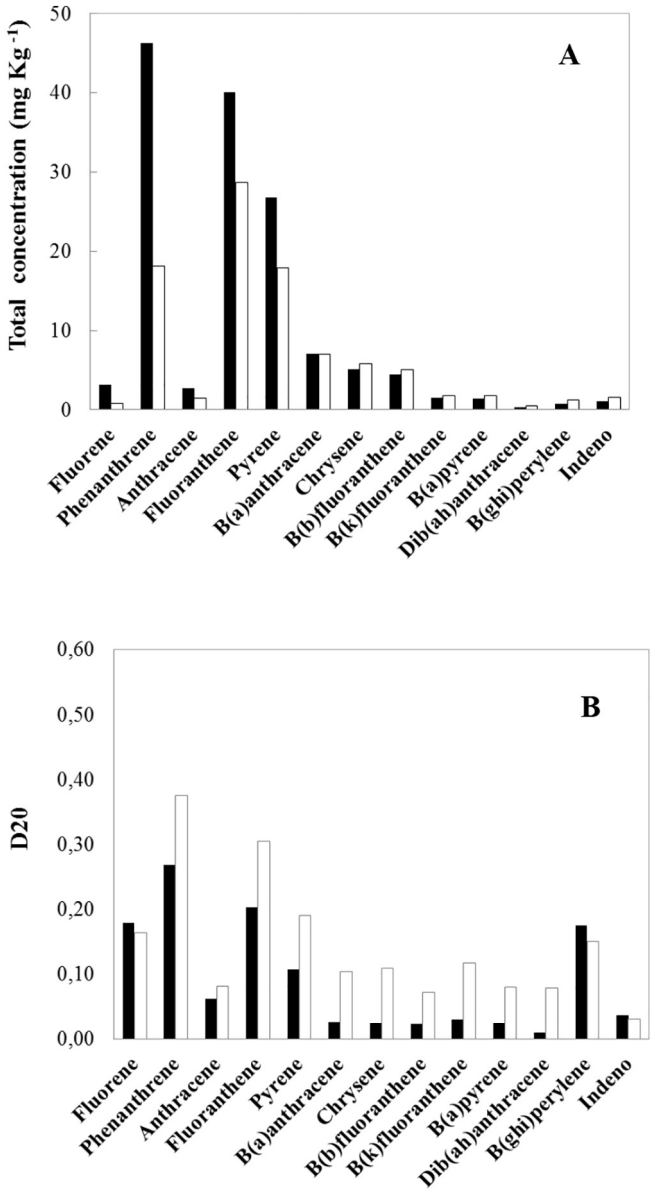

Fig. 2. Profile of total concentration (A) and D20 (fraction of PAH extracted with Tenax after 20 h) (B) in the untreated sample 8 from the area of "Ex-Carbochimica" in Fidenza, Italy (black bars) and after 60 days of bioaugmentation, sample 9 (white bars).

compared with sample 1, indicates that PAHs were less bioavailable after traditional bioremediation.

The desorption extraction method was also applied in samples that originated from a phytoremediation trial (samples 3, 4 and 5). The sample 4 was a sample originated after 60 days of phytoremediation and can also be compared to sample 3 as time-zero control. The measurement of total PAH concentrations showed a decrease by 80.4% in the sample 4 with respect to sample 3. The D20 fraction also declined during this period, indicating that biodegradation efficiently removed most of the fast desorbing chemicals. The influence of sunflowers in biodegradation of PAHs has been studied previously (Tejeda-Agredano et al., 2013; Olson et al., 2007), but we provide in this study how bioavailability evolves in the presence of the plant, what has not been covered by previous studies. Therefore, the main results obtained in these samples from traditional bioremediation indicate that the decline in total concentration was accompanied by the decline in D20 values.

3.3. Effect of bioavailability-oriented bioremediation on D20

In this section, we studied samples from bioremediation approaches addressing increases in bioavailability of the PAHs in the treated soils. For example, after phytoremediation, the effect of biosurfactant addition was also studied. This effect, together with the effect of planting sunflowers, was evident in sample 5, in which the D20 fraction for phenanthrene and benzo(a)pyrene was higher than in the sample 4 (Table 3), in spite of their similar values in total concentrations. This increase in bioavailability can be attributed to the biosurfactant. The effect of surfactants in enhancing biodegradation in PAHs contaminated soils is known (Adrion et al., 2016), but in this work the biosurfactant was applied at later stages, when most of the rapidly desorbing fraction of PAHs had decreased. This controlled increase in bioavailability, minimizing the environmental risk, is relevant in sustainable remediation frameworks (Ortega-Calvo et al., 2015; Ortega-Calvo et al., 2013). The objective in these cases is to increase bioavailability to enhance the biodegradation of slowly-desorbing PAHs. Again, D20-based assessments provided information on the bioremediation results, not directly evident through the measurement of total PAH concentrations.

The desorption extraction method was also applied in samples from bioaugmentation with a selected set of microorganisms that could potentially operate on bioavailability (samples 6–11). The promoting effect of bioaugmentation on biodegradation is known since several years ago (Vogel, 1996) and nowadays this method is used for the removal of PAHs from contaminated soil (Guo et al., 2017; Shi et al., 2018; Zafra et al., 2017; Innemanová et al., 2018; Koshlaf et al., 2019). Some of the species used in these studies were included in the consortium used in our bioaugmentation treatment such us: *Fusarium*, *Aspergillus*, *Acinetobacter calcoaceticus* and *Pseudomonas* sp. In addition to study the dissipation of the parent compounds, our focus was to test the effect of bioaugmentation on bioavailability through D20 assessments.

Table 3
Bioavailability of phenanthrene and benzo(a)pyrene in the samples described in Table 1.

Sample number	Organic carbon (%)	Clay (%)	ΣPAHs (mg kg^{-1})	Phenanthrene		Benzo(a)pyrene	
				Total concentration (mg kg^{-1})	D20[a]	Total concentration (mg kg^{-1})	D20[a]
1	7.08	24.80	4370.00 ± 331.37	843.10 ± 17.20	0.75 ± 0.05	56.50 ± 0.90	0.10 ± 0.02
2	5.53	24.80	580.00 ± 9.21	45.98 ± 9.30	0.08 ± 0.009	35.70 ± 2.50	0.07 ± 0.008
3	1.07	21.20	513.30 ± 83.70	197.10 ± 28.60	0.42 ± 0.007	4.12 ± 0.30	0.20 ± 0.01
4	1.70	21.20	100.49 ± 7.53	4.43 ± 0.34	0.055 ± 0.006	2.22 ± 0.08	0.06 ± 0.005
5	2.04	21.20	75.51 ± 14.89	6.05 ± 2.01	0.24 ± 0.08	1.81 ± 1.05	0.32 ± 0.05
6	2.03	18.10	88.43 ± 19.40	36.65 ± 8.40	0.23 ± 0.04	0.64 ± 0.21	0.03 ± 0.01
7	1.12	18.10	65.98 ± 4.28	24.87 ± 2.53	0.57 ± 0.11	0.57 ± 0.11	0.12 ± 0.04
8	0.94	39.60	140.60 ± 13.60	46.30 ± 9.10	0.27 ± 0.02	1.40 ± 0.40	0.024 ± 0.01
9	1.07	39.60	91.60 ± 5.80	18.15 ± 1.7	0.37 ± 0.03	1.70 ± 0.30	0.08 ± 0.001
10	5.77	14.50	141.17 ± 14.33	10.82 ± 0.56	0.03 ± 0.01	7.63 ± 1.75	0.07 ± 0.03
11	5.96	14.50	140.18 ± 17.92	10.10 ± 4.44	0.06 ± 0.01	9.72 ± 0.71	0.08 ± 0.01

[a] Fraction of PAH extracted with Tenax after 20 h.

Biodegradation was also observed in the sample 7 (which was originated from sample 6 after 60 days of bioaugmentation) for almost all PAHs, and it was more pronounced in mineralizable PAHs, as phenanthrene, than in the co-metabolizable PAHs, as benzo(a)pyrene. The percentage of biodegradation, calculated with respect to the initial concentration, was 32% and 11% for phenanthrene and benzo(a)pyrene, respectively (Table 3 and Fig. 3). In sample 7, the effect of bioaugmentation in biodegradation was accompanied by an increase in the D20 fraction of the two target compounds. These results show an effect of bioaugmentation on the bioavailability of PAHs. One possibility of this increase could be that in the consortium used some microorganisms would be producing biosurfactants. For example, species from *Pseudomonas* and *Acinetobacter* are well-known biosurfactant producers and inhabitants of PAHs contaminated soils (Bento et al., 2005). The efficiency of bioaugmentation depends of abiotic and biotic factors, chemical structure, concentration of the pollutants and physico-chemical properties of soil (Mrozik and Piotrowska-Seget, 2010). To our knowledge, this is the first report of a bioaugmentation-driven shift in bioavailability of the pollutants caused in soil by the inoculated microorganisms.

Similar results to those observed with bioaugmented, creosote polluted soil were obtained in the industrially polluted soil from the Italian site, also treated with bioaugmentation during 60 days (Table 3, samples 8 and 9). These results were already presented in detail in Section 3.1, but are commented here for comparison purposes with the other two bioaugmented soils. In this case, the difference was with benzo(a)pyrene, where the increase in D20 was not accompanied by the decrease in its total concentration of this compound, after 60 days. Finally, the samples 10 and 11 evidenced a lack of biodegradation after 60 days of bioaugmentation, as evidenced by negligible changes in PAH concentrations (Table 3). The initially low D20 values observed for phenanthrene and benzo(a)pyrene in sample 10, that did not change after treatment (sample 11), are consistent with the observed recalcitrance, by indicating an extremely low bioavailability of the pollutants, that would eventually resist the potential of the inoculated consortium to enhance bioavailability.

4. Conclusions

We highlighted the importance of assessing bioavailability during a bioremediation approach because this measurement provides a more risk-based information than that provided by total PAHs concentrations only. In this work the bioavailability was measured as the fraction of PAH extracted after 20 h with Tenax (ISO/TS 16751) and it is one of the first studies that uses this measurement systematically in the field of bioremediation. The single-time point Tenax extraction method has resulted a reliable and robust way to determine bioavailability of PAHs in a wide set of samples from different treatments (phytoremediation, biostimulation and bioaugmentation). However, our objective was not to generally compare the effects of these different bioremediation approaches towards PAH biodegradation or increase in PAH bioavailability, what will be the subject of further research. The soils used in this study varied in soil properties, contaminant concentrations, and location of soil collection. Some different treatments were subjected to some different soils, and it is therefore difficult to conclude that only bioaugmentation or biosurfactant stimulation (as an individual bioremediation approach) can always lead to an increased bioavailability. Nevertheless, differences often emerged through measurements of D20 between samples from traditional bioremediation and samples in which the effect of bioavailability-oriented bioremediation was studied: while the D20 value decreased in samples originated from traditional bioremediation, in samples from bioavailability-oriented bioremediation approaches, such us using biosurfactant or using an additional consortia, the D20 fraction increased. By showing the usefulness of these measurements in the bioremediation field, these results provide additional environmental scenarios for improved risk-based evaluations based on bioavailability of organic pollutants.

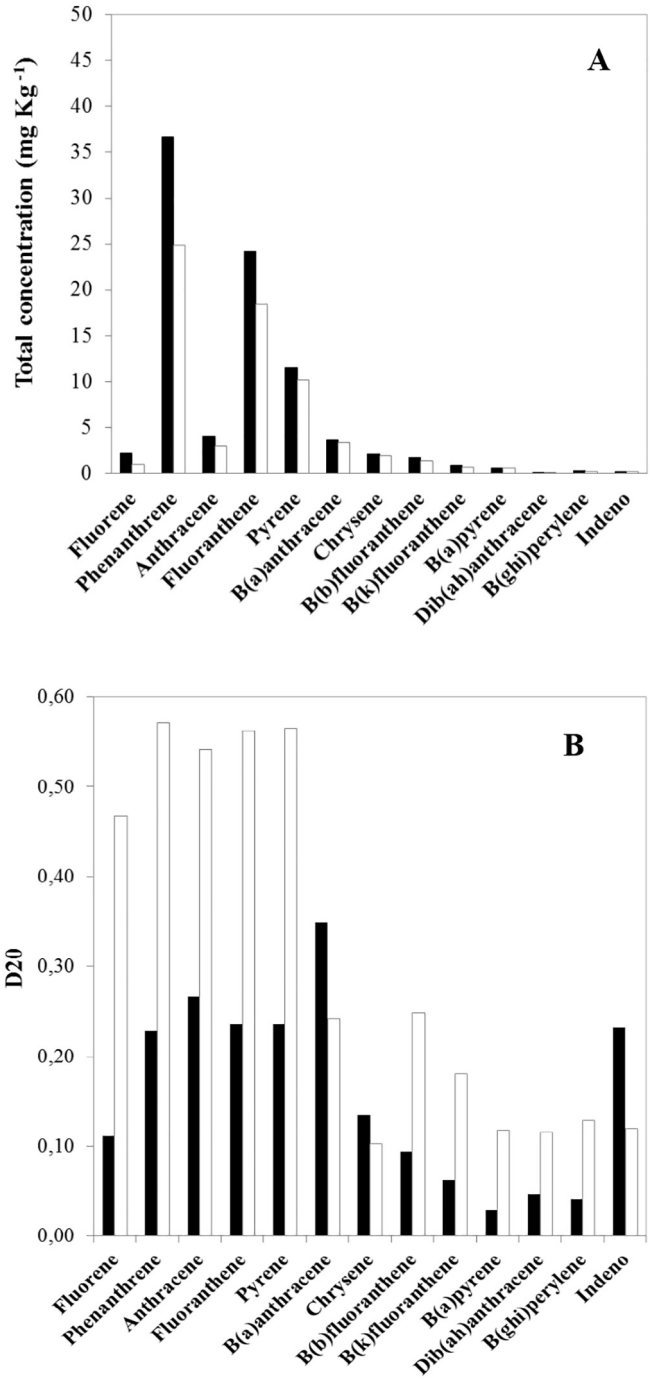

Fig. 3. Profile of total concentration (A) and D20 (fraction of PAH extracted with Tenax after 20 h) (B) for the untreated soil from Andújar, Jaén (sample 6) (black bars) and after 60 days of bioaugmentation, sample 7 (white bars).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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