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Integrating bioavailability measurements in persistence testing of partially biodegradable organic chemicals in soil

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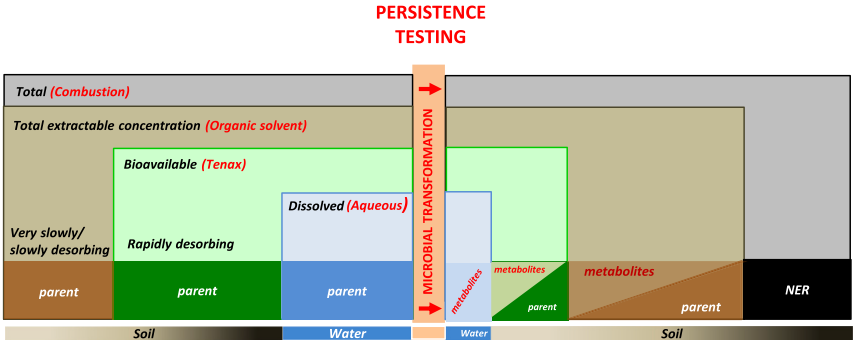
HIGHLIGHTS

- Two standardized methods (ISO16751:2020 and OECD 307) have been integrated.
- The ISO method was slightly adapted for use in more polar compounds.
- Partial transformation of pyrene decreased bioavailability.
- Carbamazepine metabolites had a high affinity for the aqueous phase.
- The results obtained have important implications in risk assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Overestimation of risk is one of the main problems in environmental risk assessments if only total concentration of organic pollutants is considered. In this study, we integrated bioavailability measurements into persistence testing of pollutants in soil to show that it is the key to have a more realistic environmental risk assessment (ERA). To this integration, two standardized methods were used: OECD 307, as persistence test, and ISO 16751: 2020, to bioavailability measurements based on 20 h extractions with a strong adsorbent (Tenax), using pyrene and carbamazepine as model test substances. Because the ISO method was initially designed for nonpolar compounds with log $K_{ow} > 3$, a slight adaptation was necessary for carbamazepine (log $K_{ow} = 2.7$), assuming this also as an extension of the applicability domain of the method. During the biodegradation of these compounds, the mineralization extents did not exceed 4 %, giving rise to transformation products. Therefore, the bioavailability measurements covered both the parent compound and the metabolites produced. In the case of pyrene, the partial transformation carried out by a specialized microbial inoculum accounted for, respectively, 32 % or 40 % of the initial concentration (4 mg kg^{-1}) in unamended or compost-amended soil. Only 1 % was present as hydrophilic transformation products that were not trapped by Tenax, but partitioned into the water. The non-bioavailable residue increased in both soils after biodegradation. The distribution of chemicals in the different phases of the system was the key to assess more realistically the shifts in bioavailability during persistence

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testing. The same procedure was carried out for carbamazepine where an additional desorption study showed a slower desorption rate of the parent compound after incubation. In this case, 25 % of transformation products was mobilized to the aqueous phase. Our results show that bioavailability measurements provide valuable information when integrated in persistence testing, therefore contributing to realism in prospective ERA scenarios.

1. Introduction

Chemical compounds are classified as persistent if they are not subject to biological, chemical, or physical degradation. It is environmentally disadvantageous if persistent chemical compounds are discharged into ecosystems, as this might lead to an accumulation of the compounds in the environment with potentially negative impacts on organisms. Persistence is a major risk standard, often measured together with toxicity and bioaccumulation, so it is an important parameter to be accounted for in environmental legislation.

Persistence is often assessed separately to bioavailability, which addresses the issue as to whether an organic pollutant exists in a physical state such that it can be taken up by organisms and causes hazard in the environment. A recent proposal highlighted the importance of introducing bioavailability assessments in the evaluation of risk and to be able to make more appropriate decisions in the management of pollution caused by organic chemicals (Ortega-Calvo et al., 2015). With the purpose of facilitating greater acceptance by regulators, Ortega-Calvo et al., 2015 proposed that slowly desorbing chemicals are not bioavailable over a significant time. This proposal was published in 2020 by the ISO Technical Committee (ISO Technical Committee, 2020). The International Standard ISO/16751 specifies a method for an approximation of the potential bioavailable and nonbioavailable fractions of organic pollutants. The potential bioavailable fraction is the amount of the pollutant in the matrix that is exchangeable with the aqueous phase - the major exposure pathway in environmental risk assessment. The method is based on the pollutant fraction which is strongly adsorbed by an adsorbent (Tenax) or a complexing agent (cyclodextrin) in a time of 20 h. This standardized method of using desorption extraction with cyclodextrin or Tenax to define the bioavailability of hydrophobic organic compounds in soils has already been employed in a variety of retrospective (Posada-Baquero et al., 2022; Posada-Baquero et al., 2019) and prospective risk assessment scenarios (Harmsen et al., 2019) which are relevant for soil quality and agricultural practices. Bioavailability measurements may be employed, for example, to support the use of locally-available soil amendments to immobilize trace elements and organic pollutants and decrease their bioavailability to soil microorganisms and plants (Rodríguez-Vila et al., 2016; Teodoro et al., 2020; Ye et al., 2019; Guo and Zhang, 2020). The use of compost amendments is a very good alternative because it can reduce the negative impacts of pollutants present in reclaimed wastewater and enhance sustainability in agricultural soils, providing circular economy principles and producing a positive effect on soil functions (Kranz et al., 2020; Agegnehu et al., 2016; Manirakiza, 2020; Zhang et al., 2022).

Although bioavailability is still not part of OECD standardized schemes, it is possible to incorporate it into the current OECD persistence simulation tests. It would be possible to assess the removal of the bioavailable fraction (however it is measured) instead of or as well as of the total amount of chemical. This approach would be relevant because microbial degradation, which is often the main dissipation route in persistence tests, operates preferentially on the most bioavailable chemicals. The OECD guidelines for the testing of chemicals is a collection of approximately 150 of the most relevant internationally agreed-upon testing methods used by government, industry and independent laboratories to identify and characterize potential hazards of chemicals. A method, described in test 307 (OECD, 2002), was designed to evaluate the aerobic and anaerobic transformation of chemicals in soil. This test aims to determine the rate at which the test substance transforms, as well as the nature and rates of the formation and decline of transformation products, which may have an impact on plants and soil organisms. With the use of ^{14}C-labelled compounds, the mineralization rates of the test substance can be measured by capturing the evolved $^{14}CO_2$. Additionally, a mass balance can be established, accounting for the formation of nonextractable residues (NER). The occurrence of partial transformation (e.g., metabolite formation) as the main dissipation route is, however, considered, from a regulatory perspective, within the remits of persistency, i.e. the transformed chemicals remain in soil as a possible source of risk, independent of whether they are bioavailable.

To our knowledge, studies combining detailed desorption extraction methods used in persistence simulation tests with soils are limited. The significance of evaluating bioavailability during a soil biodegradation test was emphasized in some studies, to check if slow degradation kinetics are due to substance persistence or are rather caused by limited bioavailability of the substance to degraders (Posada-Baquero et al., 2022; Posada-Baquero et al., 2019). Therefore, we propose that the concepts of biodegradation and bioavailability in risk assessment should be integrated with each other. The integration of bioavailability assessments in standardized procedures for monitoring the biological transformation of organic chemicals in soil would lead to more realistic assessments of persistence and risk. In our study, we examined two environmentally relevant compounds, pyrene (PYR) and carbamazepine (CBZ), in such a combined persistence and bioavailability scenario. Pyrene is a polycyclic aromatic hydrocarbon (PAH) with a relatively high level of toxicity (Juhasz et al., 2003). Anthropogenic activities, such as coal burning, coking, and automobile exhaust gas, are the main sources of PYR released into the environment (Tang et al., 2005). Our research group has worked intensively with this compound using it as an example indicator of the risk and persistence of PAHs and recent studies on the risk reduction and cometabolism of PYR in soils have been published (Fernández-López et al., 2021; Castilla-Alcantara et al., 2023). CBZ is a commonly prescribed anticonvulsant and antiepileptic pharmaceutical compound. CBZ is categorized as a medium to high-risk pollutant in the environment, and it has been detected in the soil environment with the potential for uptake into crops, involving potential hazards to human health (González García et al., 2018). For our persistence tests, we employed the two compounds as ^{14}C-labelled chemicals, and an agricultural soil that was modified to increase sorption of the pollutants and possibly reduce their bioavailability through amendment with compost. The partial transformation of PYR was guaranteed through inoculation with a soil bacterium (*Pseudomonas putida* G7) able to cometabolize the compound (Fernández-López et al., 2021), while we relied on autochthonous soil microorganisms for the partial transformation of CBZ (Li et al., 2013).

2. Materials and methods

2.1. Chemicals

[4,5,9,10-^{14}C]-pyrene (^{14}C-PYR, 58.8 mCi/mmol, radiochemical purity >98 %, dissolved in acetone) was purchased from Campro Scientific GmbH (Veenendaal, The Netherlands). Ring-labelled [^{14}C]-carbamazepine (^{14}C-CBZ, 74.5 mCi/mmol, radiochemical purity 100 %, dissolved in methanol) was obtained from Moravek Inc. (Brea, USA). ^{12}C-pyrene and ^{12}C-carbamazepine (purity 98 %) were purchased from Sigma Aldrich (Madrid, Spain). Analytical grade dichloromethane, acetonitrile, hexane, acetone and methanol were supplied by Fischer Chemical (Madrid, Spain). Tenax (60–80 mesh, 177–250 μm) was

supplied by Buchem BV (Apeldoorn, The Netherlands).

2.2. Soil

The soil used in this study was collected from the agricultural experimental station of the Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC). The soil was sieved (2 mm sieve) before use. The physicochemical properties of the soil were as follows: pH 8.44; 0.44 % organic carbon; 0.046 % organic nitrogen (Kjeldahl); 8.0 mg kg^{-1} Olsen phosphorus; 122 mg kg^{-1} available potassium; particle size distribution (Bouyoucos, 1962): 71.6 % coarse-grained sand, 6.9 % fine-grained sand, 10.6 % silt, and 10.8 % clay. The soil had a loamy-sandy texture. The concentration of native pyrene in this soil was 31.58 ± 9.55 μg kg^{-1}. Some of the experiments were performed with soil amended with compost, which was supplied by Eider (Guadix, Spain). This material originated from wooden residues and sewage sludge. The physicochemical properties of the compost were as follows: 25 % of humidity, pH 8.58; 18.1 % organic carbon; 31.2 % organic matter, and 1.28 % organic nitrogen (Kjeldahl). This compost was mixed with the soil (7 % *w*/w). The mixture has 1.63 % organic carbon and 2.8 % organic matter. The soil was sterilized for all experiments with pyrene and for the abiotic controls of carbamazepine experiments. Sterilization was performed by autoclaving with three cycles at 105 °C for 60 min each.

2.3. Spiking of organic compounds in soil

^{14}C-PYR or ^{14}C-CBZ was added to 50 g portions of soil that would later be incubated in accordance with the OCDE 307 guidelines. For radiorespirometry measurements, 100 μL of an acetone or methanol solution containing 250,000 dpm of the labelled chemical and sufficient unlabelled chemical to give a final concentration of 4 mg kg^{-1} was dispensed onto a 1 g soil subsample. For bioavailability measurements, the soil contained ten times more radioactivity to increase the sensitivity of liquid scintillation after HPLC fractionation. When the solvent was completely evaporated, this subsample was mixed with 49 g of soil (Li et al., 2013). The homogeneity of the spiked compounds was checked in 1 g subsamples from the mixtures that were combusted in an oxidizer (307 Sample Oxidizer, Perkin Elmer), and the ^{14}C content was determined by liquid scintillation in a QUANTULUS 1220 counter (PerkinElmer) with Permafluor E+ liquid scintillation cocktail (PerkinElmer); the recovery was 90 % for the two studied compounds.

2.4. Soil incubation conditions

The degradation experiments with the soils were carried out using the aerobic incubation conditions described in the OECD 307 guideline (OECD, 2002) with two modifications: i) inoculating the soil with a pyrene cometabolizing bacterium (for experiments with pyrene) and ii) the incubation temperature was 23 ± 2 °C instead of the OECD-recommended value (20 ± 2 °C). These modifications had the aim of favouring microbial transformation of the test compounds for bioavailability assessments after incubation for 12 d for pyrene and 60 d for carbamazepine, a period selected after preliminary experiments to allow for the partial transformation of the chemicals in soil.

Mineralization was measured using closed biometer flasks (Bellco glass, NJ) containing 50 g of spiked soil. Milli-Q sterilized water was added to adjust the soil moisture (40 % of water holding capacity). The production of $^{14}CO_2$ was measured, in duplicate biometer flasks, as radioactivity appearing in the alkali trap of the biometer flasks (Fernández-López et al., 2021). The radioactivity was measured by liquid scintillation counting (LSC) with a Beckman LS6500 liquid scintillation counter (Beckman Instruments, Fullerton, California, U.S.A.). The assessment of bioavailability was performed in duplicate Erlenmeyer flasks where the soil was incubated in parallel under exactly the same conditions as in radiorespirometry measurements. After incubation, duplicate flasks were kept frozen at −20 °C until they were used for extractions.

In the experiments with pyrene, the soil in each flask was inoculated with 2 mL of a suspension of *Pseudomonas putida* G7 cells. Previous research from our group has demonstrated the capability of this bacterium to degrade pyrene by co-metabolism when inoculated into this agricultural soil. The bacterium was cultivated and prepared for inoculation as described elsewhere (Fernández-López et al., 2021), to give a cell density of 10^8 cell g^{-1}. The soils had been previously sterilized as explained above. In accordance to that previous research, we did not expect any significant transformation of pyrene during the experimental period in the absence of inoculation, either through biodegradation (by the autochthonous microbial populations) or abiotically. Therefore, we considered unnecessary to run uninoculated or abiotic controls in this specific study with pyrene. With carbamazepine, the soils were not inoculated, relying on the capacity of the autochthonous soil microorganisms to transform the chemical (Li et al., 2013). In this second case, the soils were preincubated for 7 d before the addition of the compound to activate microbial activity. This preincubation process was performed with 50 % of the humidity employed in the incubations. The abiotic controls were in this case incubated and treated in the same conditions that the rest of the flasks, and remained sealed until the end of the experimental period.

2.5. Bioavailability assessments

2.5.1. Pyrene

To measure the bioavailable concentration (as mg kg^{-1}) of ^{14}C-pyrene equivalents (^{14}C-PYR$_{eq}$) in the soil, we placed 2.0 g of dry soil, 35 mL of Milli-Q water, sodium azide (200 mg L^{-1}) and 0.7 g of Tenax TA beads in 50 mL stainless steel centrifuge tubes equipped with stainless steel sealing (Heraeus-Sorvall, Madrid). The tubes were closed and shaken at 120 rpm for 20 h at 23 ± 2 °C on a rotary shaker, and then centrifuged for 10 min at 17,212*g*. The floating Tenax beads were fully recovered with a spatula and transferred into a 250 mL screw-capped Erlenmeyer flask containing 100 mL of acetone/hexane (1:1), which was kept overnight on a rotary shaker operating at 150 rpm. The beads were removed by filtration (cellulose filter, 45 μm, previously rinsed with acetone), and the organic phase was collected. An aliquot (10 mL) was mixed with 10 mL of Ultima Gold scintillation cocktail and measured by LSC to determine the total ^{14}C-PYR$_{eq}$ extracted by Tenax. The ^{14}C-PYReq concentrations were calculated by the direct translation of the total dpm counts into milligrams of chemical present as parent compound or metabolites, taking the specific activity of ^{14}C-pyrene in the soil (Fernández-López et al., 2021; Castilla-Alcantara et al., 2023). In our conditions, 1 dpm corresponded to 8×10^{-8} mg ^{14}C-PYReq.

The percentage of total ^{14}C-PYR$_{eq}$ corresponding to the parent compound (parent ^{14}C-PYR$_{eq}$) was determined by HPLC fractionation and LSC analysis of the remaining extract. For this aim, the organic solvent in the extract was rotary evaporated, and the extract was then redissolved in acetonitrile and filtered through 0.45 μm nylon filters. Fractionation was carried out with a Waters HPLC system following a chromatographic procedure described elsewhere in detail for this and other PAHs (Posada-Baquero et al., 2022). In this specific study, HPLC was coupled with a fraction collector (Waters Fraction Collector III), to allow for subsequent LSC determination of ^{14}C in five different fractions in which the eluent was collected. In accordance with its retention time (25 min), the parent compound appeared in the fourth fraction. In addition, the equipment was connected to a fluorescence detector, which allowed for parallel quantification (Posada-Baquero et al., 2022). The fractions, collected into 20 ml scintillation vials, were mixed with 10 ml of Ultima Gold scintillation cocktail and measured by LSC. The concentration of the parent compound determined by ^{14}C measurement, was in agreement (> 95 %) with the concentration determined in the same extracts by HPLC and fluorescence detection. Due to the low ^{14}C counts detected in the eluent fractions different from pyrene, the

percentage of ^{14}C-PYR$_{eq}$ that appeared as metabolites is reported as the difference between total ^{14}C-PYR$_{eq}$ and parent ^{14}C-PYR$_{eq}$.

The concentration of ^{14}C-PYR$_{eq}$ remaining in soil after Tenax extraction (i.e., the nonbioavailable concentration) was determined by exhaustive solid liquid extraction followed by combustion. The exhaustive solid liquid extraction used a subsample of 0.5 g of soil and a mixture of 100 mL of acetone/dichloromethane (1:1 *v*/v) with a Soxtherm extractor (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). This extract was subsequently treated as the extract obtained from Tenax to determine the total extractable, parent and metabolite ^{14}C-PYR$_{eq}$ concentrations in soil through HPLC fractionation and LSC. The radioactivity remaining in the soil after the complete procedure was determined by combustion in an oxidizer as described in section 2.3 and reported as ^{14}C-PYR$_{eq}$ in NER. The concentration of ^{14}C-PYR$_{eq}$ in the aqueous phase resulting from Tenax extractions was also measured by LSC in 10 mL aliquots of the supernatant after centrifugation and Tenax removal.

2.5.2. Carbamazepine

The physicochemical properties of carbamazepine (17.7 mg L^{-1} water solubility and 2.7 log K$_{ow}$) are quite different from those of pyrene (0.13 mg L^{-1} water solubility and 4.8 log K$_{ow}$), what made necessary to adapt the methodology, since the application of ISO 16751:2020 was validated for compounds with log K$_{ow}$ > 3. In this adaptation, the main difference was the Tenax/soil ratio. In this case, to measure the bioavailable concentration (as mg kg^{-1}) of ^{14}C-carbamazepine equivalents (^{14}C-CBZ$_{eq}$) in the soil, we used 1.0 g of dry soil, 35 mL of Milli-Q water, sodium azide (200 mg L^{-1}) and 1.5 g of Tenax TA beads. Some aqueous extractions (i.e., with aqueous phase only) were run in parallel to Tenax extractions, under the same conditions, with the only difference that no Tenax was added to the tubes. These tubes were treated as indicated in section 2.5.1, and the floating Tenax beads were fully recovered with a spatula and transferred into another 50 mL stainless steel centrifuge tube to be extracted following a procedure described elsewhere (Martin et al., 2010), with slight modifications. Tenax beads were extracted by sequential extraction: Tenax was initially extracted with 10 mL of methanol by sonication for 15 min followed by centrifugation at 17,212 *g* for 10 min. Sequential extractions were then performed with 5 mL of methanol, 5 ml of acetone and 10 ml of acetone/methanol (50:50 *v*/v). Each supernatant was transferred to an amber glass flask with a screw cap (these supernatants were not combined to avoid a decrease of the sensitivity of the LSC measurements). The recovery through this procedure was 97.3 ± 2.2 %. An aliquot (1 mL) from each supernatant was mixed with 5 mL UltimaGold to determine by LSC the total CBZ$_{eq}$ extracted by Tenax. The concentration of ^{14}C-CBZ$_{eq}$ was calculated, as explained above for ^{14}C-PYReq, from the specific activity of ^{14}C-carbamazepine in the soil (1 dpm corresponded to 8 × 10^{-8} mg ^{14}C-CBZ$_{eq}$). To determine the percentage of total ^{14}C-CBZ$_{eq}$ corresponding to the parent compound (parent CBZ), the extracts were combined and analysed by LSC after HPLC fractionation using the same methodology as that described for ^{14}C-PYR$_{eq}$ measurements. In this case, the chromatographic method was modified slightly: the mobile phase used was an acetonitrile/Milli-Q water gradient (with the following proportion of acetonitrile: 30 % at initial time, 50 % at 20 min, 100 % at 30 min and 30 % at 35 min), and the column was installed in a thermostatic oven at 30 °C, 1 mL min^{-1} of flow, 20 μl injection volume, and run time of 35 min. In accordance with its retention time (13 min), the parent compound appeared in the third fraction from a total of four fractions collected. In addition, parallel identification and quantification of the parent chemical was performed with a Water 996 photodiode array detector connected to the HPLC. The concentration of the parent compound determined by ^{14}C measurement was in agreement (> 95 %) with the concentration determined in the same extracts by HPLC and photodiode array detection, therefore excluding any interference caused by eventual coelution of metabolites in the same ^{14}C fraction as the parent compound. Due to the low ^{14}C counts detected in the eluent fractions different from carbamazepine, the percentage of ^{14}C-CBZ$_{eq}$ that appeared as metabolites is reported as the difference between total ^{14}C-CBZ$_{eq}$ and parent ^{14}C-CBZ. The total ^{14}C-CBZ$_{eq}$, parent chemical and metabolite ^{14}C-CBZ$_{eq}$ values were quantified in the aqueous phase resulting from Tenax extractions with the same methodology, with the difference that no previous step of extraction with an organic solvent was necessary. Due to the modified conditions for Tenax extraction of ^{14}C-CBZ, the limited soil mass used (1 g) caused difficulties in determining separately solvent-extractable and NER ^{14}C-CBZ$_{eq}$ fractions remaining in soil after Tenax extraction. Therefore, the nonbioavailable ^{14}C-CBZ$_{eq}$ concentration was determined in the soil residue by combustion in an oxidizer as described in section 2.3.

The desorption kinetics were determined for ^{14}C-CBZ in duplicate with the compost-amended soil before and after incubation through four consecutive Tenax extractions during an experimental period of 168 h. Tenax adsorbent and Milli-Q water were replaced by new water at each sampling time and the parental ^{14}C-CBZ contents were analysed both in Tenax and water as described above. The desorption data were compared to a first-order two-compartment model:

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

where S_t and S_o (mg) are the soil-sorbed amounts of ^{14}C-CBZ at time t (h) and at the start of the experiment, respectively; F_{fast} and F_{slow} are the fast- and slow-desorbing fractions, respectively; and K_{fast} and K_{slow} (h^{-1}) are the rate constants of fast and slow desorption, respectively. The total amount of parent ^{14}C-CBZ recovered experimentally from the soil through these Tenax extractions was used to calculate the value for S_o. The recovery of the nominal concentration, accounting for the chemical in the Tenax, the aqueous phase and the soil (after combustion) was, respectively, 105.5 ± 13.4 % and 96.4 ± 2.1 % for the unincubated and incubated soil.

3. Results and discussion

3.1. Effect of partial transformation on the bioavailability assessment of pyrene

Before incubation, the total concentration of ^{14}C-PYR$_{eq}$ in the soils determined independently through exhaustive extraction and combustion (Table 1) was in good agreement with the nominal concentration of ^{14}C-PYR, 4 mg kg^{-1}. Bioavailability assessments in both unincubated soils through Tenax extraction yielded different results. The bioavailable concentration of ^{14}C-PYR$_{eq}$ in pure soil was double that of the compost-amended soil, which was attributable to the significantly higher TOC content of the latter. The low concentration of ^{14}C-PYR$_{eq}$ dissolved in the aqueous phase of Tenax extractions (0.0021 ± 0.0006 μg mL^{-1} and 0.0026 ± 0.0004 μg mL^{-1} for unamended and amended soil, respectively) confirmed the capacity of Tenax to act as a sink for the soil-desorbed chemical in our system.

The unamended and amended soil was inoculated with a cometabolic-competent bacterium and incubated under OECD 307 conditions. In both the absence and presence of compost, a lag-phase was not observed (Fig. S1A) and the mineralization extent was 0.085 ± 0.03 % and 0.03 ± 0.01 % for soil and soil and compost, respectively (Fig. S1A). These low mineralization extents and the complete ^{14}C recovery by combustion after incubation (Table 1) indicate that, if it occurred, the cometabolic transformation of pyrene by the inoculum gave rise to ^{14}C-labelled metabolites that remained in the soil. The incubation led to a significant decrease in Tenax extractable ^{14}C-PYR$_{eq}$ compared to unincubated soils (Student's test, $P \leq 0.05$). The percentage distribution of the metabolites and the parent compound in each fraction is shown in Fig. 1, where the initial distribution (i.e., before incubation) is also shown for comparison purposes. The results with the unamended soil (Fig. 1, upper part right) indicate that Tenax phase efficiently trapped the metabolites (labelled a light green colour box), which

Table 1
Total and bioavailable concentrations of ^{14}C-pyrene equivalents (mg kg^{-1}) in the unamended and amended soil, before and after incubation.

			Soil		Soil + compost	
Treatment	Extraction method	Fraction	Concentration	Recovery (%)[a]	Concentration	Recovery (%)[a]
Unincubated	Combustion	Total	4.27 ± 0.10	107	4.04 ± 0.46	101
	Organic solvent	Total extractable[b]	4.34 ± 0.52	109	3.40 ± 0.14	85
	Adsorbent	Water dissolved	0.048 ± 0.005	103	0.045 ± 0.008	ND[d]
		Tenax	3.20 ± 0.26		1.57 ± 0.17	
		Non-Bioavailable	0.93 ± 0.22		2.38 ± 0.17[c]	
	Combustion	Total	4.11 ± 1.40	104	3.6 ± 0.2	ND[d]
	Organic solvent	Total extractable[b]	1.95 ± 0.87	49	3.24 ± 0.13	81
After 12 d biodegradation	Adsorbent	Water dissolved	0.12 ± 0.02	72.5	0.03 ± 0.0003	74
		Tenax	2.17 ± 0.12		0.73 ± 0.05	
		Non-Bioavailable	0.61 ± 0.24		2.19 ± 0.37	

[a] Recovery of the nominal concentration (4 mg kg^{-1}), as indicated in the text;
[b] Results obtained after extractable fraction from the bulk soil;
[c] Concentration calculated by difference from the nominal concentration;
[d] ND, not determined.

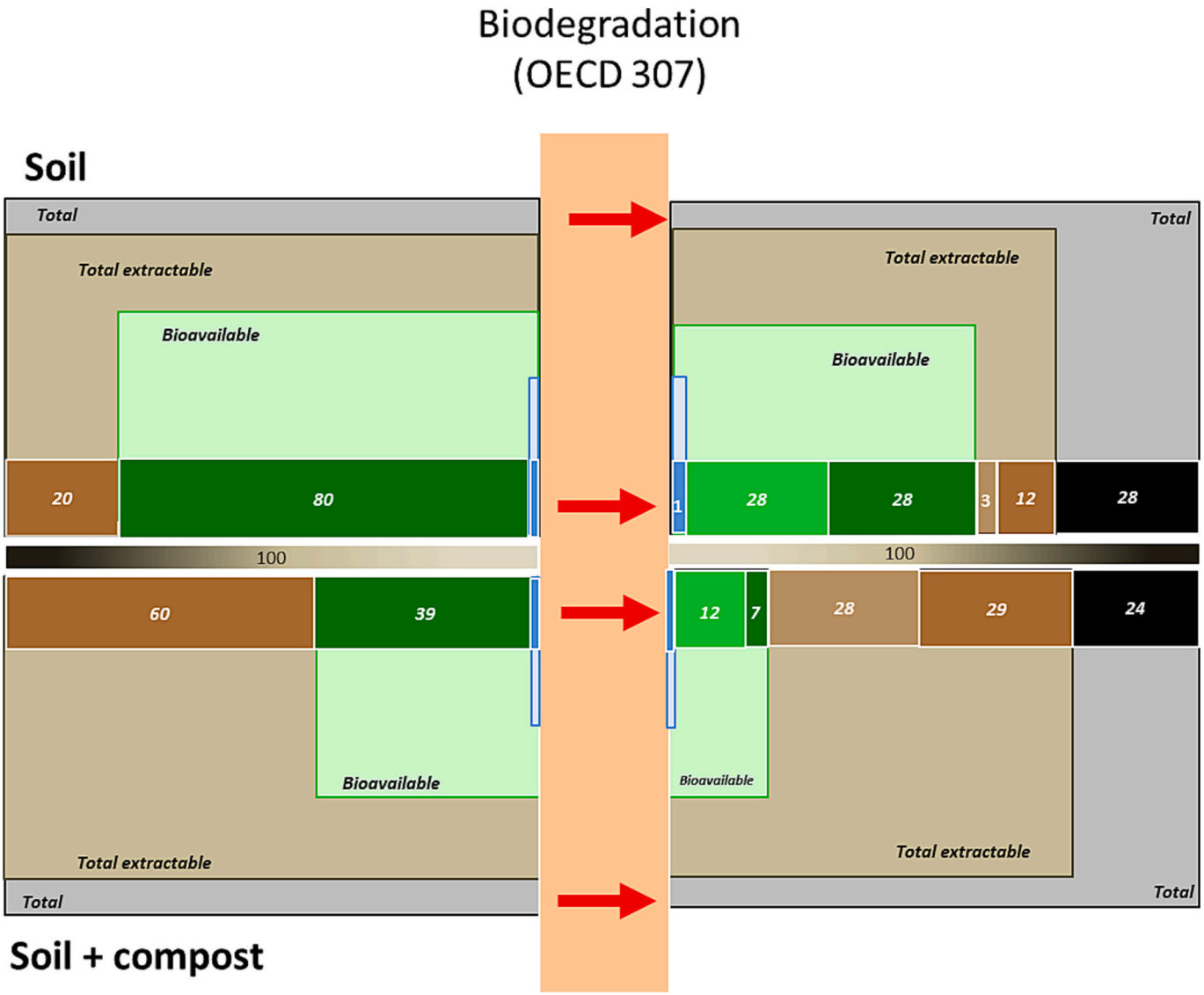

Fig. 1. Percentage distribution of pyrene and its metabolites (both as ^{14}C-PYR$_{eq}$) in each phase of Tenax extractions of soil (upper part) and compost-amended soil (lower part) to assess bioavailability, before and after the OECD 307 incubation test. Green, brown and black boxes indicate, ^{14}C-PYR$_{eq}$ detected in Tenax, extracted from soil with organic solvents, and remaining in soil as nonextractable residue or NER, respectively. Light green and light brown colours indicate ^{14}C-PYR$_{eq}$ detected as metabolites, whereas the respective darker colours indicate the parent chemical. The fraction of chemical dissolved in the aqueous phase is shown in blue.

accounted for 50 % of the bioavailable ^{14}C-PYR$_{eq}$, and left behind a minor fraction of the extractable metabolites in the soil (light brown colour). Only 40 % of the parent compound was detected after incubation both in Tenax and in the soil (dark green and dark brown boxes, respectively), with the remaining ^{14}C-PYR$_{eq}$ present as Tenax- or solvent-extractable metabolites or as NER (black box). With the compost-amended soil (Fig. 1, lower part right), the transformation decreased the bioavailable fraction of the parent chemical to 7 % of the initially present compound. In contrast to the unamended soil, the metabolites partitioned only partially to Tenax, remaining in the soil as nonbioavailable but solvent-extractable ^{14}C-PYR$_{eq}$.

The significantly different bioavailable concentrations of parent PYR observed in the two different soils after incubation (Fig. 1) exemplify how bioavailability measurements provide very useful information when integrated into persistence assessments. While persistence was very similar in the two soils (40 % and 36 % of the initial PYR concentration was found in unamended and amended soils, respectively), bioavailability was reduced significantly in the compost-amended soil after incubation. A similar result was found with the metabolites extracted by Tenax. These results suggest that a comparable persistence in dissimilar environmental media may result in different environmental risks if we consider bioavailability. Furthermore, persistence in a given environment does not necessarily imply a risk if the remaining chemical has a limited potential for mobilization into the water phase.

3.2. *Effect of partial transformation on the bioavailability assessment of carbamazepine*

Radiorespirometry determinations showed in nonsterile soils a low level of mineralization after 60 d, as only 3.55 ± 0.5 % and 1.04 ± 0.01 % of the initially present ^{14}C-CBZ was transformed into $^{14}CO_2$ in the soil and compost-amended soil, respectively (Fig. S1B). There was also a low level of mineralization in the abiotic control, with <0.1 % of the compound mineralized in both soils (Fig. S1C).

In contrast to pyrene, the bioavailability assessment of ^{14}C-CBZ through Tenax extraction, before incubation, left a significant fraction of the chemical dissolved in the water phase, both with the soil and with the compost-amended soil (Table 2). The resulting bioavailable fraction, accounting for the water dissolved and Tenax phases, was slightly lower in the amended soil. Indeed, the extraction with only water yielded basically the same bioavailable concentration as the Tenax extraction method (i.e., calculated as the sum of concentrations in the water and Tenax). However, the bioavailable fraction of ^{14}C-CBZ$_{eq}$ decreased slightly in both soils after incubation, with concomitant decreases in the water and Tenax phases in the unamended and compost-amended soils, respectively. Sterilization of the soils only prevented the change in phase distribution of ^{14}C-CBZ$_{eq}$ observed after incubation in the case of the unamended soil. The compost-amended soil showed a similar ^{14}C-CBZ$_{eq}$ distribution among phases, with or without sterilization.

The analysis of the parent compound and metabolites in the water and Tenax phases of unincubated and incubated soils revealed significant differences. Their percentage distribution among different phases is shown in Fig. 2. In both incubated soils, a significant fraction of ^{14}C-CBZ$_{eq}$ was present as metabolites in both phases, and accounted for approximately 40 % of the parent chemical originally present, thus confirming that the transformation had occurred. Furthermore, the water phase contained mostly metabolites, whereas the parent compound partitioned preferentially into Tenax. The aqueous concentration of parent ^{14}C-CBZ in these Tenax extractions was 0.023 μg mL^{-1} and 0.010 μg mL^{-1} for unamended and amended soil, respectively. The fraction of the parent compound trapped by Tenax was higher in the unamended soil, which was agreement with its lower TOC content. The relative occurrence of radioactivity observed as metabolites after HPLC fractionation of the extracts from the inoculated treatment in amended soil suggested, at least qualitatively (due to the low counts in these fractions, which made the reliable quantification of these fractions difficult), a different polarity of the metabolites that partitioned to Tenax from those that were present in the aqueous phase. In Tenax extract, most of the radioactivity was present in metabolite fractions with a lower polarity than ^{14}C-CBZ (i.e., eluted afterwards), whereas in the water fraction, most of the metabolite radioactivity moved to more polar compounds. This is in concordance with another study about the metabolites of carbamazepine detected during degradation in soil, which widely differ in hydrophobicity, such as hydroxylated derivatives and acridine (Li et al., 2013). No metabolites were detected in Tenax extractions before incubation and incubated, sterilized soils, which evidenced the microbial nature of the transformation observed in incubated soils.

Table 2
Total and bioavailable (Tenax and dissolved) concentrations of ^{14}C-carbamazepine equivalents (mg kg^{-1}) in the unamended and amended soil, before and after incubation.

			Soil		Soil + compost	
Treatment	Extraction method	Fraction	Concentration	Recovery (%)[a]	Concentration	Recovery (%)[a]
Unincubated	Combustion	Total	5.00 ± 0.30	125	4.60 ± 0.30	115
	Adsorbent	Water dissolved	1.53 ± 0.15	93	1.02 ± 0.09	94.25
		Tenax	1.90 ± 0.16		2.16 ± 0.04	
		∑ [b]	3.44 ± 0.30		3.18 ± 0.13	
		Non-Bioavailable	0.28 ± 0.09		0.59 ± 0.13	
	Aqueous	Dissolved	3.32 ± 0.12	87	3.20 ± 0.06	ND[c]
		Non-bioavailable	0.15 ± 0.01		0.82 ± 0.06[d]	
After 12 d biodegradation	Combustion	Total	3.70 ± 2.06	92.5	3.98 ± 0.21	99.5
	Adsorbent	Water dissolved	1.22 ± 0.04	93	0.98 ± 0.04	ND[c]
		Tenax	2.02 ± 0.19		1.86 ± 0.16	
		∑ [b]	3.24 ± 0.23		2.84 ± 0.20	
		Non-Bioavailable	0.48 ± 0.08		1.16 ± 0.12[d]	
	Aqueous	Dissolved	2.82 ± 0.001	ND[c]	ND[c]	
		Non-Bioavailable	1.17 ± 0.02[d]			
Abiotic control	Combustion	Total	4.50 ± 0.09	112.5	ND[c]	ND[c]
	Adsorbent	Water dissolved	1.49 ± 0.15	85	0.73 ± 0.12	ND[c]
		Tenax	1.78 ± 0.06		1.85 ± 0.16	
		∑ [b]	3.27 ± 0.21		2.58 ± 0.28	
		Non-bioavailable	0.13 ± 0.01		1.42 ± 0.27[d]	

[a] Recovery of the nominal concentration (4 mg kg^{-1}), as indicated in the text;
[b] Sum of extracted ^{14}C-CBZ$_{eq}$ in Tenax and dissolved in the water phase;
[c] ND, not determined;
[d] Concentration calculated by difference from the nominal concentration.

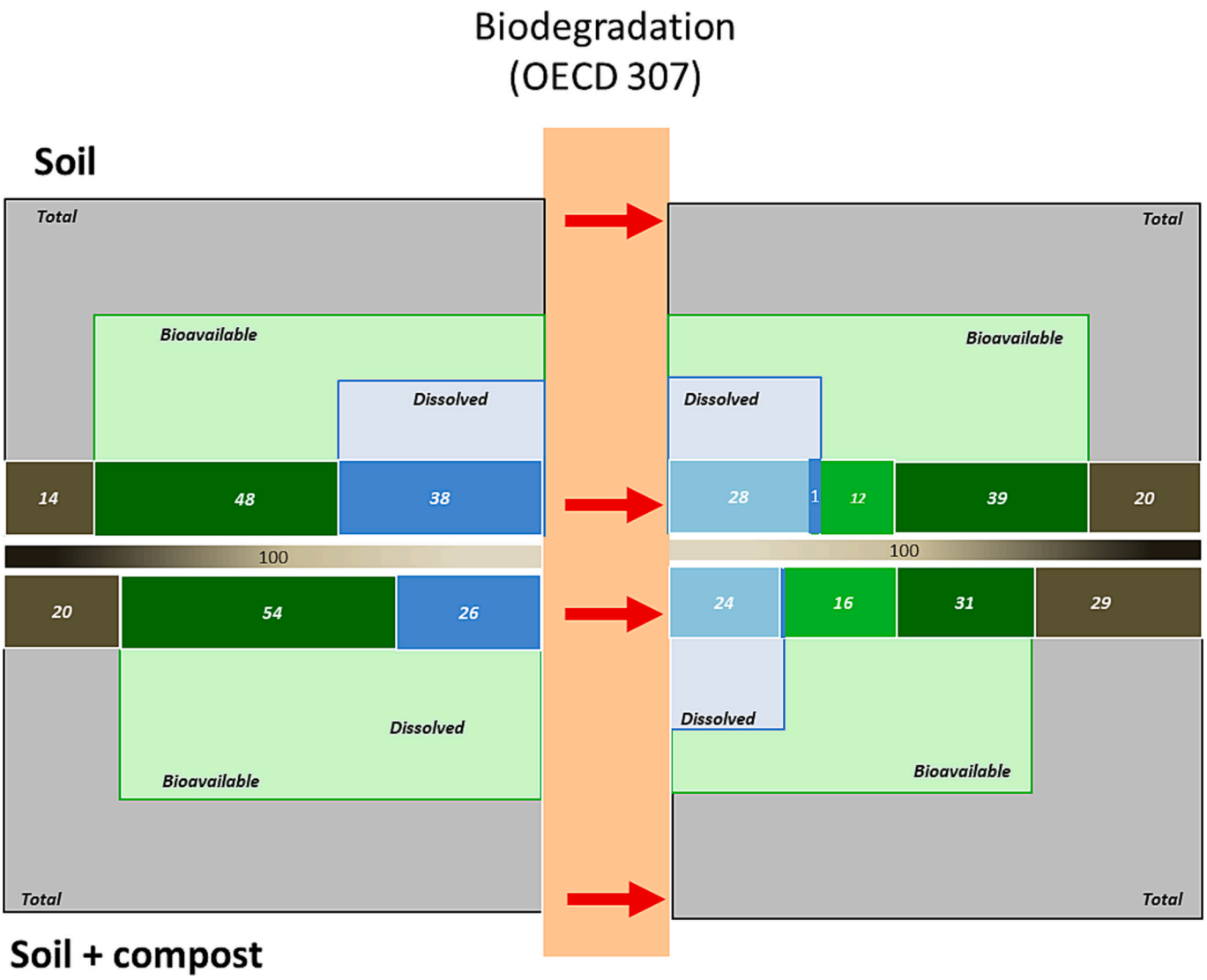

Fig. 2. Percentage distribution of carbamazepine and its metabolites (both as ^{14}C-CBZ$_{eq}$) in each phase of Tenax extractions of soil (upper part) and compost-amended soil (lower part) to assess bioavailability, before and after the OECD 307 incubation test. Blue, green, and grey boxes indicate, respectively, ^{14}C-CBZ$_{eq}$ detected in the aqueous phase, in Tenax, and remaining in soil as nonbioavailable fraction. Light blue and light green colours indicate ^{14}C-CBZ$_{eq}$ detected as metabolites, whereas the respective darker colours indicate the parent chemical.

In parallel to the preferential partitioning of the parent CBZ into Tenax in incubated soils, the relative polarity of the metabolites, qualitatively evidenced after HPLC fractionation, indicated, as expected, that the more polar metabolites remained in the water phase, whereas Tenax adsorbed more hydrophobic transformation products. With pyrene, most of the desorbed metabolites were found in Tenax phase, similar to the parent chemical. These results indicate that the modified ISO method could be employed not only to assess bioavailability of the parent contaminants, but also aiding in the determination of the potential environmental risks from the metabolites formed as a result of their partial transformation. Furthermore, the incorporation of the water phase into the quantitative assessment would provide a more complete picture. These approaches would be useful in the nontarget analysis of pollutants and their metabolites that generate risk through the water phase, therefore extending the applicability domain of the ISO method based on desorption extraction.

3.3. Effect of incubation on the kinetics of desorption of parent carbamazepine

The observed change in the concentration of water-dissolved ^{14}C-CBZ in Tenax extractions of the soil before and after incubation could be explained by a change in the rate of desorption of the chemical. Therefore, the desorption kinetics of ^{14}C-CBZ were determined in these two scenarios (Fig. 3). Eventual interferences caused by ^{14}C-CBZ metabolites in liquid scintillation measurements on incubated soils were avoided through previous HPLC fractionation and measurement of ^{14}C in the fraction containing the parent compound only. The figure indicates that the biphasic model (Eq. 1) allowed for sufficient prediction of ^{14}C-CBZ desorption, both in the unincubated soil and in the incubated soil. The dashed lines in the figure represent the model results with the mean values of duplicate experiments. Kinetic analysis of ^{14}C-CBZ desorption showed that, without incubation, F_{fast} accounted for 80 ± 6 % of the initially present chemical, with a K_{fast} value of 0.24 ± 0.03 h^{-1} (yielding a half-life for fast desorption of 3 h) and a K_{slow} value of 3 ± 1 10^{-3} h^{-1}. The kinetics of desorption changed significantly after incubation, especially through a decreased rate of fast desorption, resulting

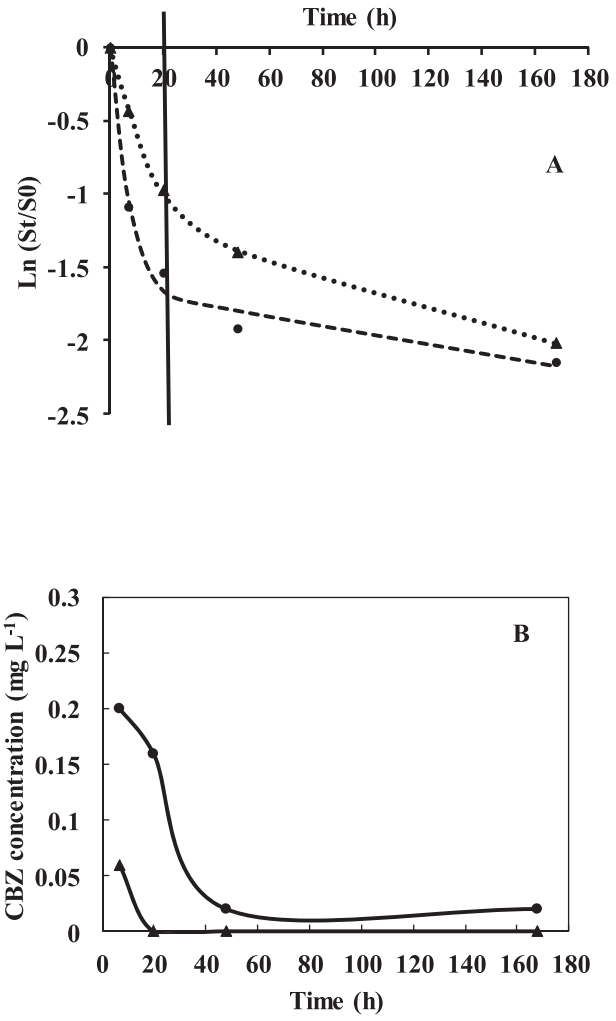

Fig. 3. Desorption kinetics of ^{14}C-labelled carbamazepine from compost-amended soil determined by Tenax extraction. A, time evolution of parent ^{14}C-CBZ concentration in soil (as Ln S_t/S_0 – see text) during Tenax extraction before (circles) and after (triangles) 60 d of incubation. The dashed lines represent the model fitting desorption results to Eq. (1). B, Time evolution of ^{14}C-CBZ concentration in the aqueous phase at each time point from panel A.

in a longer half-life of 7 h (F_{fast} = 70 ± 0.03 %; K_{fast} = 0.1 ± 0.001 h^{-1}, K_{slow} = 5.0 ± 0.7 10^{-3} h^{-1}). Furthermore, the aqueous-dissolved ^{14}C-CBZ concentration reached undetectable levels after 20 h during Tenax extraction of incubated soils (Fig. 3B), ensuring the infinite-sink assumptions (i.e., the adsorption to Tenax was fast enough to drive the desorption kinetics from soil to its maximum through the maintenance of a very low aqueous concentration) (Van Noort et al., 2014; Birdwell and Thibodeaux, 2009). The vertical line in Fig. 3 shows that the operational 20 h time cut recommended by the ISO 16751 method for a single-point extraction allows for the desorption of most ^{14}C-CBZ in the incubated soil present in F_{fast} (85 %), and a fraction of the chemical in F_{slow} (9 %).

The well-known performance of parent PYR in Tenax extractions (Posada-Baquero et al., 2019; Gomez-Lahoz and Ortega-Calvo, 2005) made us consider unnecessary a detailed study of the desorption kinetics for this compound to verify infinite-sink assumptions in bioavailability measurements during persistence tests. However, the log K_{ow} of CBZ is slightly below the minimum value (log K_{ow} = 3) recommended by ISO for bioavailability estimations of nonionic chemicals through desorption extraction (ISO Technical Committee, 2020). This could be considered a methodological disadvantage if a lower hydrophobicity influences the capacity of the extractant to act as an infinite sink for desorbed CBZ. Before incubation, the modified ratio of sorbent to soil used in our procedure was not sufficient to overcome this limitation, as evidenced by the presence of a relatively high proportion of CBZ in the aqueous phase. After 60 d of incubation, the low concentration of the dissolved parent chemical in Tenax extraction indicates that the slower desorption inputs into the aqueous phase were overwhelmed by Tenax removal. A plausible explanation for the change is the decrease in the desorption rate from soil after incubation, which was confirmed for the compost-amended soil by the determination of the desorption kinetics. We suggest that during the 60 d of incubation, pollutant ageing and the preferential biodegradation of CBZ initially present in the fast-desorbing pool led to a shift in desorption that made possible the trapping of F_{fast} within the 20 h time window proposed by the ISO method. This explanation agrees with previous observations on the change in desorption kinetics of PAHs in the presence of biological activity in soil and sediment (Posada-Baquero et al., 2019; Gomez-Lahoz and Ortega-Calvo, 2005), enriching slowly desorbing chemicals. Furthermore, these results indicate that the desorption extraction method can be extended to bioavailability assessment of less hydrophobic chemicals, in situations where they have been in the soil or sediment for sufficient time for the ageing and biodegradation of the more labile fractions to occur.

3.4. Environmental implications

PAHs are relevant soil contaminants and meanwhile far more PAHs than the well-established 16 EPA PAHs are subject of human and environmental risk assessment. An overview on the currently discussed substances is given elsewhere (Achten and Andersson, 2015). The methodology used with pyrene in this study could in principle be applied to all PAHs which can also be biodegraded partially through co-metabolism, such as benzo[*a*]pyrene (Wang et al., 2019; Haritash, 2009; Chibwe et al., 2017; Rentza et al., 2005). Pharmaceuticals are soil contaminants of emerging concern (Keerthanan et al., 2020), and the results obtained with carbamazepine could have implications for many other pharmaceutical compounds. For example, antibiotics such as sulfamethoxazole, sulfamethizole and trimethoprim can be partially transformed through cometabolic reactions by a variety of bacteria (*Rhodococcus rhodochrous, Aspergillus niger* and *Pseudomonas* sp.) (Gauthier and Cooper, 2010; Alvarino et al., *2016*). Another example is paracetamol, a pain medicine, where transformation routes in soil (Li et al., 2014) and even biochar amended soils (Chacón et al., 2022) are well investigated. The information from our approach would be very useful to study the bioavailability of the transformation products during biodegradation, what may be relevant in a variety of situations where biologically-enhanced environmental risk is considered, such as the bioremediation of PAH-polluted soils, or in the prospective risk assessment of new pharmaceuticals.

Our results may have also implications for rapid risk assessments performed in soils managed with a variety of agricultural practices, such as the soil amendment with locally-available materials and the irrigation with reclaimed wastewater. In this study, the amendment with compost had a clear effect on reducing the desorption of the pollutants from soil after the incubation and consequently their bioavailability, decreasing the risks in comparison with the unamended soil, supporting that it is a very good option for real agricultural practice. Our results showed no major impact of compost addition on the extent of biodegradation because the incubation period allowed biodegradation to act mainly on the fast desorbing pool, where there would have not been major differences with and without amendment. In addition to showing the usefulness of these bioavailability measurements in persistence assessments, the application of these methods in these management scenarios constitutes a reliable and robust way to quickly evaluate agricultural operations involving soil amendments.

4. Conclusion

We highlighted the importance of assessing bioavailability during a persistence evaluation of chemicals in soil because this measurement provides more risk-relevant information than that provided by total concentrations of the parent chemical only. There is a paucity of studies comparing Tenax extractions of a wider variety of chemicals and environmental scenarios, and on the use and applicability of the standardized OECD 307 persistence test. To our knowledge, this is the first report of bioavailability changes during persistence tests in which a partial transformation of the chemicals occurs, i.e., not leading to mineralization. The two model compounds, pyrene and carbamazepine, underwent significant transformation into metabolites that remained in the soil. The method allowed us to determine a change in bioavailability after incubation, as well as dissimilar partitioning of the parent compound and metabolites into the water phase. With pyrene, the transformation led to lowering the risk due to ageing and the formation of non-desorbable metabolites, whereas with carbamazepine, the remaining parent chemical desorbed more slowly, but the metabolites showed a high mobility in water, therefore contributing to risk. Therefore, our study shows that more information is obtained when bioavailability measurements are integrated into persistence assessments which allows us to make a more realistic environmental risk assessment.

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CRediT author statement

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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.

Data availability

No data was used for the research described in the article.

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