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In Vitro Bioaccessibility of Soil-Bound Polycyclic Aromatic Hydrocarbons in Successive Digestive Compartments in Cows

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Ruminants, which have a central place in the food chain, ingest soil that may contain pollutants. The bioaccessibility of three different polycyclic aromatic hydrocarbon compounds from soil was studied using an in vitro model based on the digestive tract of cows. For this purpose, pasture soil was spiked with ^{14}C radio-labeled compounds, aged, and then exposed to conditions which simulated the digestive compartments of the rumen, abomasum, and intestines. Our results show that aging generally reduced the bioaccessibility of all the compounds tested. Total bioaccessibilty in the first digestive compartment, i.e., the rumen, depended on the considered compound: elevated for phenanthrene (17–24%), moderate for pyrene (6.6–8.1%), and low for benzo[*a*]pyrene (2.3–3.6%). Bioaccessibility was very low in abomasal acidity (generally <2%) and intestinal colloids (<8%). The liquid phases of intestinal medium successfully extracted compounds from freshly contaminated soil (25–28%), but the bioaccessibilty dropped markedly after aging (17% for phenanthrene and <9% for the more lipophylic compounds). Total bioaccessibilty in this in vitro model ranged from 11% for benzo[*a*]pyrene in aged soil to 58% for phenanthrene in freshly contaminated soil, and the bioaccessibility of this latter compound was always higher compared to pyrene or benzo[*a*]pyrene. Residual soil contained around half of the initial load, the highest residual levels being of benzo[*a*]pyrene, which confirms the observed bioaccessibility.

KEYWORDS: PAH; soil; bioaccessibility; in vitro model; ruminants

1. INTRODUCTION

Increasing occurrences of polycyclic aromatic hydrocarbon (PAH) pollution in developed countries have attracted the attention of the scientific community. Although low transfer rates of soil-bound PAHs to milk have been demonstrated in cows (*1*), milk from lactating ruminants grazing near anthropogenic PAH sources may provide significant human exposure to PAHs. These authors suggested the extraction of compounds from the soil matrix prior to absorption and transfer to milk during passage through the digestive tract. This mobilization of contaminants in soil during digestion is generally defined as bioaccessibility (*2*). However, the role of the different digestive compartments in cows in this extraction process has not yet been studied. Some studies focusing on monogastric models (*2–5*) considered the global bioaccessibility of different pollutants, but without making a distinction between different digestive compartments. The role of the first and most active digestive compartment in ruminants (in our case, the rumen) regarding the extraction of soil-bound PAHs is still unknown. This lack of knowledge is particularly problematic because, first, the absorption of different compounds in the rumen is generally understood and, second, soil intake by cattle averages around 0.5 kg per day at pasture and may even reach 1.5 kg (*6*, *7*). Insofar as soil contamination levels of about 100 mg/kg of soil have been reported (*8*, *9*), cattle may thus be highly exposed to soil-bound pollutants on contaminated sites. Another issue relates to the hydroxylation of parent compounds during the digestive process. Methods based on native compounds only may neglect metabolized forms. Indeed, the hydroxylation of PAHs by soil bacteria has been reported (*10–12*) and might enhance the rising proportions of metabolized compounds when contact time with the soil increases. Moreover, metabolization by microorganisms in the rumen can be expected.

The aim of this study was thus to compare the extraction rates of different soil-bound PAHs in the main digestive compartments of an in vitro ruminant model. The use of isotope-labeled compounds [^{14}C] enabled measurement of the global behavior of the various chemical forms of the compounds considered.

2. MATERIALS AND METHODS

Soil was artificially spiked with three radiolabeled PAHs. It was aged as described below and then subjected consecutively to three in vitro treatments to simulate different digestive compartments: the rumen,

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Table 1. Soil Characteristics Prior to Spiking

characteristic	value
mineral matter	86.9
clay (<2 μm), %	32.0
silt (2–50 μm), %	54.4
sand (>50 μm), %	13.6
organic matter (g/kg)	13.1
total N (g/kg)	0.856
ratio C/N	8.88
exchange capacity (mmol/kg)	86.8
pH	6.04

Table 2. Characteristics and Contents of the Selected Compounds

	phenanthrene (Phe)	pyrene (Pyr)	benzo[*a*]pyrene (BaP)
CAS number	85-01-8	129-00-0	50-32-8
molecular weight (g/mol)	178.2	202.3	252.3
no. of benzene rings	3	4	5
lipophilicity (log K_{ow} at 25 °C)	4.6	5.2	6.5
water solubility at 25 °C (mg/L)	1.3	0.14	0.0038
no. of jars used	1 and 2	3 and 4	5 and 6
native content (μg/kg of soil)[a]	43.9	32.6	19.4
radiolabeling content after spiking and aging	9-^{14}C	4.5.9.10-^{14}C	7.10-^{14}C
mg/kg of soil	5.4	6.8	8.4
mCi/kg of soil	1.67	1.67	2.02

[a] Used method NF X 33-012 (AFNOR).

abomasum, and intestine. Bioaccessibility of the considered compound was compared in terms of either the partial accessibility within each considered compartment or the total accessibility as a ratio of the soil load prior to any incubation.

2.1. Soil Preparation. The A horizon (to a depth of less than 5 cm) of soil from a permanent pasture was sampled, cleaned of plant roots, air-dried (2 weeks at 15 °C), and sieved (2 mm). The soil characteristics are presented in **Table 1**. Fifty grams of the prepared soil was placed in six separate jars. Three PAH compounds were then chosen to model differences in extraction as a function of the molecular weight, number of benzene rings, and the lipophilicity profile of the compound considered: phenanthrene (Phe), pyrene (Pyr), and benzo[*a*]pyrene (BaP). The use of radiolabeled compounds (^{14}C) enabled overall monitoring of the native compounds and their metabolites. After the soil samples were humidified with distilled water (80% of their water holding capacity), each compound was used to fortify two different jars using 2 mL of acetonitrile as the vector compound. The doses used are shown in **Table 2**.

After contamination, the freshly spiked soil was aged for 1 or 30 days at 15 °C, in darkness, and at constant humidity. After being aged and before incubation, the soil was stored at −18 °C.

2.2. Preparation of Solvents. Three different extraction solvents were studied: rumen liquid, stomach acid, and intestinal liquid. Spiked soil was exposed to ruminal conditions in vitro. In order to avoid any quenching due to nontransparent solvents, natural rumen juice was replaced by a widely used artificial medium according to Russell (*13*). This medium was supplemented with ruminal bacteria. To achieve this, the ruminal content was sampled in cannulated cows (#2C, Bar Diamond, Parma, ID) and transferred rapidly and under anaerobic conditions to the laboratory. The liquid phase was pressed (50 μm pore size) to separate particles from the liquid phase and bacteria were isolated by two-phase centrifugation: first at 150*g* for 10 min, and then the supernatant was centrifuged a second time at 10000*g* for 30 min. The resulting bacterial pellet was dissolved in the artificial Russell medium, and 20 mL of this mixture was inoculated into each replicate. This model enables bacterial growth for approximately 6 h (*14*).

The stomach compartment was simulated using its acid. Intestinal conditions were achieved using a mixture of phosphate buffer (to raise the pH to 8), pancreatic salts (P 1750, Sigma, Steinheim, Germany), and ox gall (B 3883, Sigma), as suggested by van der Wiele (*5*).

2.3. Incubation Procedure. One gram of spiked soil was incubated for 6 h with 90 mL of supplemented ruminal liquid at 38 °C under anaerobic conditions and constant gentle shaking. Tests were performed on two replicates at each factor level, for example, three PAH compounds and two aging levels. The proportions between soil and liquid corresponded approximately to in vivo conditions, that is, 0.5 kg of soil for an adult cow with a 90 L rumen.

Following this incubation under ruminal conditions, the residual soil was separated from the liquid phases by centrifugation at 4000*g* for 30 min. The resulting pellet was incubated for 2 h at 38 °C with 18 mL of hydrochloric acid (2 N) in order to attain a pH of 2 and thus simulate gastric conditions. The supernatant was then separated from the residual soil by centrifugation again at 4000*g* for 30 min.

Finally, the extraction was carried out in the intestinal compartment by incubating the residual soil with 140 mL of the intestinal mixture (see above) for 3 h at 38 °C. The intestinal solvent was then separated from the residual soil by decantation during 5 min. The supernatant was gently transferred to centrifugation tubes where dissolved bile salts were spun down by centrifugation at 4000*g* for 30 min. The centrifugation pellet was considered as intestinal colloids and the supernatant as the intestinal liquid.

2.4. Radioactive Monitoring. Measurements of extracted radiolabeled compounds were performed in solvents (water, ruminal liquid, stomach acid, or intestinal liquid) by liquid scintillation (Ultima Gold, Perkin-Elmer, Villebon-sur-Yvette, France) in a β-counter (Tri-Carb 2100TR, Packard Instrument Company Inc., Warrenville, IL). The obtained values were multiplied by the corresponding volume in order to quantify the total extraction of PAHs, that is, 90 mL for ruminal liquid, 8 mL for hydrochloric acid, and finally 140 mL for intestinal liquid. Radiocounting, in soil before incubation and in colloids and in residual soil after all incubation steps, was carried out following complete oxidation (950 °C for 1.5 min) in a Packard 307 sample oxidizer (Packard). The $^{14}CO_2$ thus released was trapped as HCO_3^- in Carbosorb-E and filled using a Permafluor-E scintillation cocktail. The radioactivity of the samples was then established as described above.

2.5. Calculations and Statistical Analysis. The bioaccessibility of studied compounds by the different solvents was calculated in two complementary ways: First, the total bioaccessibility was the ratio of the dissolved compound to its level in soil before any incubation. Second, the partial bioaccessibility expressed dissolved compounds as a ratio of the compounds contained in the soil at entry into the simulated compartment; that is, the amount dissolved in the previous compartments was sustained as shown in **Table 3**.

Two types of statistical analysis were then applied to the data. First, the partial accessibility in consecutive digestive compartments was compared by analysis of variance using the GLM procedure of SAS (version 9.1, 2004, SAS Institute Inc., Cary, NC). Each PAH compound studied was analyzed in a separate model that took account of the two levels of aging, the four digestive compartments (rumen, stomach, intestinal liquid, and intestinal colloids), and their interactions.

Second, the total accessibility of the three PAH compounds but also their contents in residual soil were compared using analysis of variance with the GLM procedure of SAS (version 9.1, 2004). This time, the analysis was carried out separately for each aging level. The model took account of compartment levels (rumen, stomach, intestinal liquid, intestinal colloids), the PAH compound (Phe, Pyr, BaP) and interaction between the factors.

In both cases, treatment comparisons were based on least-squares means using a multiple *t* test adjusted with Tukey. A 5% threshold was fixed to determine significance.

3. RESULTS

All the determined bioaccessibilities are presented in **Tables 4–6** as well as in **Figure 1**. Under both aging levels, comparisons between compounds indicated a higher total bioaccessibility for Phe than for BaP or Pyr (**Table 4**).

Table 3. Calculations for Total and Partial Bioaccessibilities in the Different Digestive Compartments

digestive compartment	total bioaccessibility, %	partial bioaccessibility, %
rumen	$\frac{^{14}\text{C/mL of supernatant} \times 90\text{ mL}}{^{14}\text{C/g of initial soil}} \times 100$	
abomasum	$\frac{^{14}\text{C/mL of supernatant} \times 18\text{ mL}}{^{14}\text{C/g of initial soil}} \times 100$	$\frac{^{14}\text{C/mL of supernatant} \times 18\text{ mL}}{^{14}\text{C/g of soil after ruminal incubation}} \times 100$
intestinal colloids	$\frac{^{14}\text{C/mL of separated colloids}}{^{14}\text{C/g of initial soil}} \times 100$	$\frac{^{14}\text{C/mL of separated colloids}}{^{14}\text{C/g of soil after abomasal incubation}} \times 100$
intestinal liquid	$\frac{^{14}\text{C/mL of supernatant} \times 140\text{ mL}}{^{14}\text{C/g of initial soil}} \times 100$	$\frac{^{14}\text{C/mL of supernatant} \times 140\text{ mL}}{^{14}\text{C/g of soil after abomasal incubation}} \times 100$

Table 4. Effects of Compound and Digestive Compartment on the Total Bioaccessibility of Soil-Bound Polycyclic Aromatic Hydrocarbons

effects		digestive compartment	least-squares means[a] Phe	Pyr	BaP
aging: 1 day		rumen	24.4 c	6.6 d	3.6 d
compartment	$P < 0.0001$	abomasum	3.5 d	1.5 d	0.8 d
compound	$P < 0.0001$	intestinal colloids	4.3 d	5.5 d	7.6 d
interaction	$P < 0.0001$	intestinal liquid	25.4 c	25.1 c	28.1 c
root MSE[b]	3.05	total extracted	**57.6 a**	**38.6 b**	**40.0 b**
aging: 30 days		rumen	16.7 b	8.1 c	2.3 c
compartment	$P < 0.0001$	abomasum	1.3 c	0.7 c	0.6 c
compound	$P < 0.0001$	intestinal colloids	3.1 c	5.6 c	5.5 c
interaction	$P < 0.005$	intestinal liquid	6.8 c	3.0 c	2.3 c
root MSE[b]	2.25	total extracted	**27.9 a**	**17.4 b**	**10.8 b**

[a] Different letters within a given aging level indicate a difference at the threshold of significance of $P < 0.05$. [b] MSE: mean square error.

Table 5. Effects of Compound and Aging on Partial Bioaccessibility in Different Digestive Compartments

effects		aging level (days)	least-squares means[a] Phe	Pyr	BaP
		rumen			
compound	$P < 0.0001$				
aging	NS	1 (fresh)	24.4 a	6.6 c	3.6 c
interaction	$P < 0.08$	30 (aged)	16.7 ab	8.1 bc	2.3 c
root MSE[a]	2.45				
		abomasum			
compound	$P < 0.0001$				
aging	$P < 0.001$	1 (fresh)	4.6 a	1.6 b	0.8 b
interaction	$P < 0.001$	30 (aged)	1.6 b	0.8 b	0.6 b
root MSE[a]	0.31				
		intestinal colloids			
compound	NS				
aging	NS	1 (fresh)	6.0	6.0	7.9
interaction	NS	30 (aged)	3.8	6.1	5.7
root MSE[b]	2.48				
		intestinal liquid			
compound	$P < 0.01$	1 (fresh)	35.2 a	27.3 a	29.3 a
aging	$P < 0.0001$	30 (aged)	8.3 b	3.3 b	2.4 b
interaction	NS				
root MSE[b]	2.21	average	**21.8 a**	**15.3 b**	**15.9 b**

[a] Different letters indicate a significant difference between means at the threshold of $P < 0.05$. [b] MSE: mean square error.

Before aging, the bioaccessibility in ruminal liquid decreased in line with the lipophilicity of the compound: from 24% for Phe to less than 4% for BaP. Abomasal acidity solubilized only a small proportion of the compounds in vitro, with a slightly but not significantly higher extraction for Phe than for Pyr and BaP. Further along the digestive tract, the compound did not significantly affect total bioaccessibility by intestinal liquid and colloids. Nevertheless, a nonsignificant trend could be noted: higher levels of lipohilicity of the compound slightly decreased accessibility under acid conditions but did not affect the intestinal bioaccessibility of soil-bound compounds after solubilization in previous compartments. Thus, intestinal liquid solubilized around one quarter of the compounds contained in soil after the effects of ruminal liquid and stomach acidity, but without any significant difference between compounds (**Table 4**).

Table 6. Effects of Compound and Aging on the Proportion of Compounds Remaining in the Soil after All Incubation Periods

effects		aging level (days)	least-squares means[a] Phe	Pyr	BaP
compound	$P < 0.05$				
aging	$P < 0.01$	1 (fresh)	42.2 b	45.3 b	52.7 ab
interaction	NS	30 (aged)	52.5 ab	51.5 ab	59.7 a
root MSE[b]	3.19	average	**47.4 b**	**48.4 b**	**56.2 a**

[a] Different letters indicate a significant difference between means at the threshold of $P < 0.05$. [b] MSE: mean square error.

Similar differences were generally observed in aged soil, but total bioaccessibility was significantly lower when compared to nonaged soil (**Figure 1**). The bioaccessibility of Phe was significantly higher, being nearly double that than of BaP and Pyr (**Table 4**). The most marked reduction in total bioaccessibility caused by aging was observed for intestinal liquid, which fell from about 25% (nonaged soil) to less than 7% after 30 days of aging (**Figure 1**, **Table 4**). No significant differences were observed between solubilization by acidity or intestinal solvents. Nevertheless, Phe once again tended to be solubilized twice as much in aqueous phase (i.e., acidity and intestinal liquid) than Pyr and BaP. A reversed but slight trend was observed in colloids (higher proportions of BaP and Pyr than Phe), but the differences still remained far from the threshold of significance.

The second part of the analysis demonstrated the effects of aging and PAH compounds on partial bioaccessibilty in vitro within a given digestive compartment (**Table 5**).

The partial bioaccessibility of soil-bound PAHs by acidity and intestinal liquid decreased significantly with aging. Aging did not significantly affect the bioaccessibilty in the rumen and

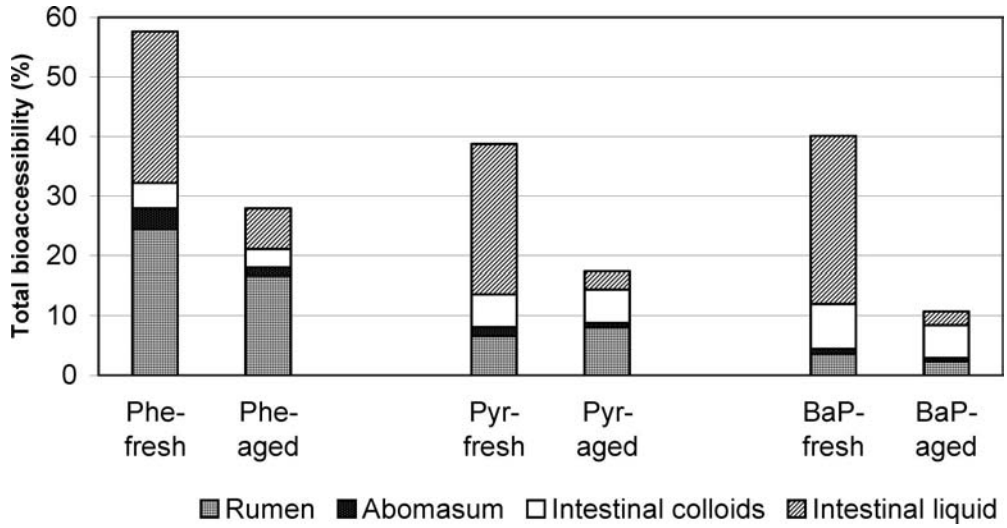

Figure 1. Total bioaccessibility on average of phenanthrene, pyrene, and benzo[*a*]pyrene depending on digestive compartment and aging level.

intestinal colloid compartments, although a nonsignificant trend toward lower Phe extraction was also observed with aged soil. The bioaccessibilities of more lipohilic Pyr and BaP did not differ in soil aged for 1 or 30 days in the rumen or intestinal colloids (**Table 5**).

In aqueous solvents, the partial bioaccessibility of Phe was significantly higher than those of Pyr and BaP. This effect was more pronounced in the first digestive compartment (i.e., rumen) than later on (i.e., abomasum or intestines). In the two first digestive compartments, aging reduced the difference in solubilization between compounds. Nevertheless, no significant difference between the partial bioaccessibilities for the three PAHs studied was observed in intestinal colloids (**Table 5**).

At the end of all incubation periods, the soil still contained about half of the PAHs initially introduced and at the same proportions as they had resisted all solvents under in vitro conditions (**Table 6**). The highest proportion of nonextracted compounds was observed in soil contaminated with BaP. The residual proportions in soils contaminated with Phe or Pyr were significantly lower than in those contaminated with BaP (**Table 6**). Moreover, nonaged soil contained significantly smaller proportions of the studied compounds than soil aged prior to incubation.

4. DISCUSSION

The levels of each compound in the soil after spiking ranged from 5 to 10 mg/kg (**Table 2**), which corresponded to a high level of contaminations when compared to the values reported in the literature (*5, 8, 9, 15, 16*) or referred to in standards (*17, 18*).

The bioaccessibility of all compounds decreased as the contact times between pollutants and soil became longer, thus confirming observations reported in the literature (*19–22*). Indeed, this aging effect allowed compounds to migrate within soil aggregates, reducing their bioavailability (*23, 24*). The impact of aging may vary considerably depending on environmental conditions, but organic matter content and particle size in the soil, as well as temperature and humidity, appear to be the principal determinants (*22, 25, 26*).

Generally speaking, bioaccessibility of Phe in soil was higher than that of BaP. Differences in chemical properties, that is, molecular weight, lipophilicity and water solubility, enhanced the higher solubilization of Phe when compared to BaP in mainly aqueous mediums. Bioaccessibility of Pyr was intermediate in the rumen and as low as that of BaP under acid conditions. It appears that the intestinal environment gave rise to similar extraction rates for all three compounds.

The bioaccessibility determined during this study was much higher than those reported with monogastric models. In highly contaminated soils (up to 200 μg/g), Hack and Selenka (*4*) observed BaP extraction rates as high as 21%. Others noted generally low bioaccessibility in similarly contaminated soils: van der Wiele (*5*) recorded a bioaccessibilty of only 2% of PAHs (mixture of 16 compounds) and Tang et al. (*16*) recorded a solubilization of around 10% of Pyr but nearly nothing for Phe or BaP.

Several hypotheses may explain the higher extraction rates that we observed in the ruminant model: first, the rumen compartment significantly increased extraction, mainly of less lipophilic compounds such as Phe. Moreover, an elevated adsorption activity of ruminal microorganisms can be supposed. Rehmann (*27*) showed that the highly lipophilic surface of bacteria would favor the adsorption of compounds extracted from soil. During our experiments, ruminal liquid was centrifuged at the end of this incubation step and it is likely that the bacteria were spun down with residual soil in the pellet. In this way, the compounds released would migrate on the bacterial surface from the rumen to the intestine before being dispersed by bile salts and subsequently counted in the intestinal supernatant or colloids. Finally, the study of labeled compounds would take account not only parent compounds but also their metabolized forms.

An interaction appears to exist between PAH compounds and the succession of digestive compartments, affecting their degree of bioaccessibility. Thus differences between the compounds mainly influenced solubilization in the upper compartments of the digestive tract, principally the rumen. Indeed, the more efficient extraction of less lipophilic compounds such as Phe was evident in the aqueous milieu of rumen, which in our model constituted the first compartment. This higher bioaccessibilty of Phe fell below the threshold of significance in subsequent compartments such as the abomasum or intestines. It is probable that the first compartment extracted the least adsorbed molecules of each compound. This action was particularly effective when the soil was freshly contaminated (i.e., not aged) and the compound considered was less lipophilic (Phe rather than BaP). Subsequent digestive compartments could possibly complete extraction by only attacking more solidly bound compounds not affected previously.

The high bioaccessibility of PAHs in the intestinal medium, and mainly before aging, was surprising when compared to monogastric models (*2*, *5*, *16*, *28*), even more so because these compounds had resisted in previous compartments. The high pH in this compartment could release compounds previously extracted from soil and then adsorbed on particles or ruminal bacteria, as suggested above. If this hypothesis can be confirmed by further studies, the effect of symbiosis with microorganisms in the rumen on the release of lipophilic pollutants would constitute a marked distinction between ruminants and monogastric animals. In monogastrics, Tang et al. (*16*) explained the increased bioaccessibility in intestines of mainly very lipophylic compounds with a strong effect of bill constituents and the formation of micelles. Our results did not confirm this observation in ruminants.

Determination of the bioavailability of PAH compounds in ruminants requires accurate data concerning the absorption of compounds after their extraction from contaminated soil. The transfer rates of parent compounds to milk are negligible, but their metabolites have been detected at higher levels (*1*, *29*). Nevertheless, recovery rate in milk does not appear to constitute the best indicator of bioavailability as the urinary excretion of these compounds has been reported to be more relevant (*30*).

Another question concerns the extrapolation of in vitro results to in vivo conditions. The model employed here did not allow for bacterial growth for longer than 6 h (*14*), and digestive transfer through the rumen is known to be much longer (*31*), at least for roughage particles. Less is known about the transfer time of very small particles such as soil, and such data would enable the improved adaptation of in vitro models. A clearer understanding of absorption and biotransformation is thus necessary if we want predict bioavailability from bioaccessibility.

In conclusion, this in vitro digestive model for ruminants showed that the bioaccessibility in freshly contaminated soil was 40–50% of PAHs. When the soil was aged, the bioaccessibility fell to less than 30% for Phe and less than 20% for strongly lipophilic compounds such as Pyr or BaP. The highest proportions of compounds were solubilized from the soil in the first compartment, that is, the rumen, and also in intestinal liquid. The highly acidic conditions in the abomasum did not enable the solubilization of notable proportions of pollutants in vitro, thus confirming previous results obtained in monogastric models. It is possible to suggest that the extraction of pollutants from soil occurs in a digestive compartment, which differs from that of their absorption, and the pollutants thus extracted are transferred between compartments adsorbed on bacteria.

However, the role of the rumen as the first digestive compartment for extraction was clearly demonstrated, especially for less lipophilic compounds. In vitro models assessing the bioavailability of pollutants for ruminants should take into account their digestive specificities. The ability of microorganisms to transform native compounds after solubilization into metabolites underlines the need of specific models to assess correctly the impact of these animals on the food chain.

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