Environ. Sci. Technol. **2011,** *45,* 1127–1132

Mobilization of Soil-Bound Residue of Organochlorine Pesticides and Polycyclic Aromatic Hydrocarbons in an in vitro Gastrointestinal Model

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Received July 30, 2010. Revised manuscript received December 1, 2010. Accepted December 6, 2010.

A previous study on mobilization of organochlorine pesticides (OCPs) in contaminated soils from the field revealed that the total amount of OCPs measured in digestive fluid and chyme of an in vitro gastrointestinal model was higher than the quantity directly extracted using a solvent extraction without digestion, providing a clue that the bound residue of OCPs might be mobilized. This hypothesis was tested in this study for both OCPs and polycyclic aromatic hydrocarbons (PAHs). Three contaminated surface soil samples with different organic carbon (OC) contents were collected from the field, and extracted with a solvent with and without digestion in an in vitro gastrointestinal model. It was found that bound residues of OCPs and PAHs were mobilized to a certain extent during digestion. The ratios of the mobilized bound residues over the total quantities extracted after digestion (R_b) varied from 0 to 0.96 for individual compounds. The R_b was positively correlated with OC content. Among the five constitutes of digestive juice, bile salt was the only one that served to mobilize the bound residues and the extractability of bile salt was constant over a concentration range from 2 to 20 mg/mL. The mobilization process followed typical first-order kinetics. The calculated rate constants suggest that mobilization was fast and 90% of extracted bound residues of OCPs and PAHs were mobilized within 2.4 and 4.8 h, respectively.

Introduction

Humans are exposed to toxic contaminants through inhalation, ingestion, and dermal contact, of which oral ingestion is often the most important pathway for hydrophobic organic pollutants (*1–3*). Soils in China are severely polluted with various contaminants, including PAHs, DDXs, and HCHs (*4–7*). As a result, in addition to food ingestion, unintentional ingestion of soil could be important in terms of oral exposure in China and other developing countries. This is particularly true for children in rural areas as a result of the poor sanitary conditions (*8*). It was estimated that the unintentional ingestion of soil by children in developed countries is from 50 to 200 mg per day (*9, 10*), and higher values are surely expected for those in developing countries due to poor sanitation conditions.

Contaminants in orally ingested soil have to be mobilized before they can be absorbed by intestinal epithelium. It is known that not all contaminants can be released from the soil matrix into the fluid during the assimilation, and exposure risk of the contaminants depends on how much can be mobilized in the digestive tract (*11*). Oral bioaccessibility of contaminants in soils can be evaluated by in vitro gastrointestinal digestion, which mimics the food digestion processes and serves as a rapid screening method (*12–18*).

In one of our previous studies, mobilities of OCPs in soils were investigated using an in vitro gastrointestinal model (*19*). It was noticed that the total quantity of α-HCH measured in the supernate and pellet after the digestion was higher than that measured in the raw samples using solvent extraction. It was thus suspected that a part of bound residue of α-HCH in the soil, which was not extractable even by a solvent extraction, was mobilized during the in vitro digestion (*19*). If this hypothesis is true, and the phenomenon is common, some sequestered pollutants in soil or other matrices such as food can be mobilized and become bioaccessible in the gastrointestinal tract.

Soil-bound residue is defined as "nonextractable pesticide residue remaining in fulvic acids, humic acids, and humin fractions after exhaustive sequential extraction" (*20*). It is generally believed that the bound residue is not mobile because the contaminants are sequestered in the soil organic matrix (*21*). However, it was found that even the bound residue could become bioavailable under certain conditions. Therefore, the possible release and delayed environmental impact of the bound residues are also of a great concern (*22, 23*). It was reported that nonextracted atrazine, isoproturon, and dicamba residues in several soils could be absorbed to earthworm tissues (*24*). It was also found that a considerable fraction of nonextractable PAH residues in a soil could be mineralized under different ecological stress conditions (*25*). To the best of our knowledge, potential mobilization of soil-bound residue of hydrophobic organic contaminants (HOCs) in animal digestive tracts has not been reported.

The main objective of this study was to test the following hypothesis: a significant amount of bound residue of HOCs, unextractable by solvent extraction, can be mobilized by gastrointestinal digestion. The HOCs studied included 4 HCH isomers (α-HCH, β-HCH, γ-HCH, and δ-HCH), 6 DDXs (*o,p′*- and *p,p′*-DDT, DDD, and DDE), and 16 parent PAHs (USEPA priority pollutants) including naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLO), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLA), pyrene (PYR), benz[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenz[a,h]anthracene (DahA), benzo[g,h,i]perylene (BghiP), and indeno[1,2,3-cd]pyrene (IcdP).

Methodology

Soil Samples. Three samples were selected from 304 surface soil samples (0−10 cm) collected from the heavily contaminated North China Plain in 2004 (*5*). The criterion for sample selection was to have different organic carbon (OC) contents and similar levels of the studied contaminants. The OC contents of these soils were 0.18 (soil A), 0.77 (soil B), and 1.46% (soil C), respectively.

Reagents. Acetone, *n*-hexane, and dichloromethane in analytical grade from Beijing Reagent, China were purified by distillation. Mixed standards of PAHs and organochlorine

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10.1021/es1025849
Published on Web 12/22/2010

pesticides (OCPs) including HCHs and DDXs, several deuterated PAHs, and other surrogates or internal standards were from J&K Chemical, U.S.A. Granular anhydrous sodium sulfate from Beijing Reagent, China was heated at 650 °C for 6 h. Silica gel of 100−200 mesh from Qingdao Marine Chemical, China was baked at 450 °C for 4 h and activated at 130 °C for 16 h prior to use. All glassware was cleaned in an ultrasonic cleaner and heated at 400 °C for 6 h. All enzymes used in the gastrointestinal model were from Sigma, USA except α-amylase was from WAKO, Japan.

Gastrointestinal Digestion Model. The in vitro gastrointestinal model was similar to the one used by Goni et al. (*26*). In brief, 2 g of soil sample was incubated in 10 mL of simulated gastric fluid at 40 °C for 2 h, at 37 °C for 12 h with the addition of 60 mL of simulated intestinal fluid, and at 37 °C for 10 h with 5 mL of simulated α-amylase digestive fluid introduced. The simulated gastric fluid was a HCl-KCl buffer solution with 50 mg/mL pepsin. The simulated intestinal fluid was a phosphate buffer with 10, 20, and 20 mg/mL pancreatin, lipase, and bile salt, respectively. The simulated α-amylase digestive fluid was a Tris-HCl buffer with 50 mg/mL α-amylase. Since the purpose of this study was to test the hypothesis rather than to measure the mobilized fractions, a slightly lower fluid/solid ratio (35:2, compared with 6.2:0.3 used by Goni et al.) was used to increase the concentrations of the target contaminants in the fluid for a better measurement (*26*). As such, artificially high bioaccessibilities could be resulted. The digestion was conducted in Teflon centrifuge tubes on a rotator (100 rpm) in dark. After incubation, the liquid and residue were separated by centrifugation (7600*g*, 10 min, Sigma 3K15, German) at 4 °C.

Digestion. Four sets of experiments were conducted. (1) To validate the mobility of the bound residue, 4 replicates with digestion (test group) and 4 replicates without digestion (control group) were extracted for each of the three soil samples. PAHs and OCPs in the digested (both solid and liquid) and the raw (without digestion) samples were measured and compared. (2) To investigate the effects of various constitutes in digestive fluid on the extractability, soil sample B was digested using five combinations: bile salt only, bile salt + lipase; bile salt + pancreatin; bile salt + lipase + pancreatin; and all the five components based on the results of a preliminary experiment (Supporting Information). For each combination, 4 replicates were conducted. Another 4 replicates added with water only were included as controls. (3) To address the dependence of the extractability on bile salt concentration, soil sample B was digested using bile salt at various concentrations of 0, 2, 5 10, and 20 mg/mL, respectively. Four replicates were conducted for each concentration. (4) To characterize mobilization kinetics, soil sample B was digested using bile salt of 10 mg/L for 0, 1, 1.5, 2, 3, 6, 14, and 24 h, respectively. Three replicates were performed for each time interval. In addition, to test the possible degradation of HOCs during digestion, the digestive solution was directly spiked with standard mixtures of HCHs, DDXs, and PAHs. The recoveries of various compounds after the digestion varied between 84 and 118%, showing no apparent degradation during the digestion (paired Wilcoxon test, $p > 0.05$).

Sample Extraction and Cleanup. The raw soil samples without digestion and the separated digestive solids from the digested samples were extracted using a microwave-accelerated reaction system (CEM Mars Xpress, USA). Twenty mL of mixed *n*-hexane and acetone solution (1:1, v/v) was used for each sample (2 g). The temperature was increased to 100 °C in 10 min, and held for 10 min at power of 1200 W. The separated supernatants were extracted twice with 30 mL of *n*-hexane for 3 min each time in a 250-mL separating funnel (with 100 mL of sodium sulfate added) on a shaker (300 rpm). For the test group, the extracts from both solids and fluid were combined. The extracts were evaporated to near dryness under reduced pressure at 35 °C in a rotary evaporator, transferred to a silica gel column (30 cm ×10 mm i.d.), and eluted with 25 mL of *n*-hexane (discarded) and 50 mL of a mixture of dichloromethane and *n*-hexane (2:3 v/v) in sequence at a rate of 2 mL/min. The eluate was concentrated to approximate 1 mL using rotary evaporation and nitrogen blow-down after being spiked with internal standards.

Sample Analysis. An Agilent gas chromatograph 6890 coupled with a HP-5 column (30 m × 0.32 mm i.d. × 0.25 μm film thickness) was used. A ^{63}Ni-ECD detector and a HP 5973 mass selective detector (MSD) were used for analyses of OCPs and PAHs, respectively. The samples were injected in splitless mode with a venting time of 0.75 min. For PAHs, the temperature was programmed from 60 to 300 °C at a rate of 5 °C/min, and then held isothermal for 20 min. The MSD was operated at 70 eV and the ion source temperature was 280 °C. Selected ion monitoring mode was performed. For OCPs, the injector and detector temperatures were 220 and 280 °C, respectively. The oven temperature was held at 50 °C for 1 min, increased to 150 at 10 °C/min, then to 240 at 3 °C/min, and finally maintained for 15 min. Soil OC content was measured by a TOC analyzer from Shimadzu 5000A, Japan.

Quality Control. Reagent and procedure blanks were included in all test series and all measurements were blank-corrected using arithmetic means of all procedure blanks. Two to four duplicates were used for all experiments to check for reproducibility, and the average coefficients of variation of the replicate samples were 13.4 and 16.8% in the fluid and 11.6 and 23.7% in the solid for OCPs and PAHs, respectively. The detection limits (calculated as 3 times the background noise for each compound) based on a 3-g soil sample were 0.01−0.06 ng/g for HCHs, 0.04−0.43 ng/g for DDXs, and 0.11−0.29 ng/g for PAHs. For the 16 spiked PAHs, the recoveries were from 72 to 121% in fluid and from 67 to 139% in residue. The recoveries for the spiked OCPs were 68 to 110% in fluid and from 67 to 120% in residue. In addition, the samples were also spiked with a range of deuterated PAHs (ACY-$_{d10}$, ACE-$_{d10}$, ANT-$_{d10}$, CHR-$_{d12}$, and perelyne-$_{d12}$) and 4,4′-dichlorobiphenyl as surrogates and the recoveries for the surrogates varied from 82 to 121%. Quantification was achieved using an internal standard method with 2-fluoro-1,1′-biphenyl and p-terphenyl-$_{d14}$ for PAHs and 2,4,5,6-tetrachloro-m-xylene for OCPs as internal standards.

Data Analysis. Statistica (v5.5, StatSoft) was applied for conducting hypothesis tests with a significant level of 5%. The results are presented as means and standard deviations.

Results and Discussion

Mobilization of Bound Residues of PAHs and OCPs. In a previous in vitro gastrointestinal digestion experiment, the total amount of α-HCH extracted after digestion seemed to be higher than that extracted directly using solvent without in vitro digestion (*19*). The possible mobilization of the bound residue in the in vitro gastrointestinal model was tested in this study using HCH-, DDX-, and PAH-contaminated soils with different OC contents. The general trends were similar among the three soils and the result for soil B is presented in Figure 1 as an example. The open circles in the figure represent the extracted quantities using conventional extraction (microwave accelerated, *n*-hexane and acetone) without digestion. The stacked bars are the total quantities in the digestive fluid and chyme after in vitro gastrointestinal digestion and extraction. For many compounds investigated, the total quantities measured with digestion were significantly higher than those without (*t*-test, $p < 0.05$). The exceptions included *o,p*′-DDE, *p,p*′-DDT, ACY, FLO, ANT, BaA, and DahA.

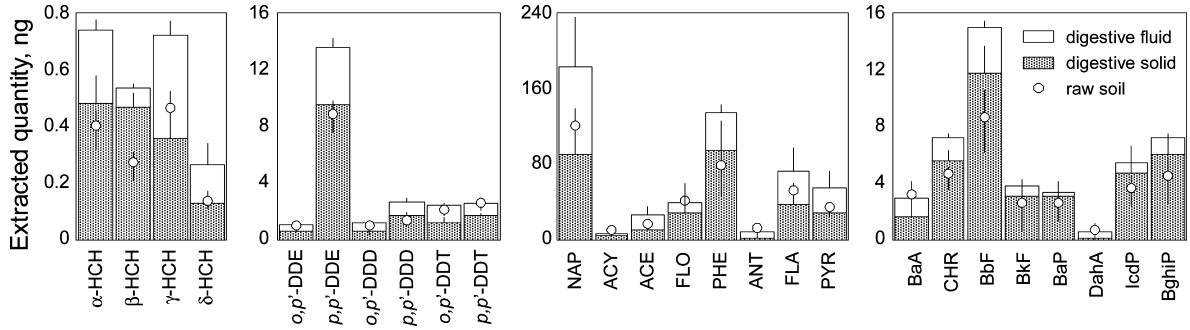

FIGURE 1. Extracted quantities of HCHs, DDXs, and PAHs from soil B with (bars) or without (open circles) the gastrointestinal digestion. Those extracted after digestion are shown as the sums of those in fluid and chyme fractions (stacked bars). The results are presented as arithmetic means and standard deviations.

It was likely that these compounds were metabolized during the experiments, leading to reduced concentrations of these PAHs. The transformation of various PAHs and OCPs in gastrointestinal digestive tract has been reported previously (*27, 28*). This is the first piece of experimental evidence showing the mobilization of the bound residue in soil and the hypothesis can be further tested by digesting solvent-extracted sample residues in the future.

To quantify the bound residue that became mobilized, a ratio of the extracted bound residue (R_b) was operationally defined as:

$$R_b = \frac{Q_{bound}}{Q_{unbound} + Q_{bound}}$$

where Q_{bound} represents the quantity of bound residue which cannot be extracted by solvent extraction directly but can be mobilized by gastrointestinal digestion and then extracted by solvent extraction, $Q_{unbound}$ is the quantity of unbound chemicals which can be extracted by solvent extraction directly without gastrointestinal digestion. For soil B, the averaged R_b of all compounds was 0.33, ranging from 0 for ACY and BaA to 0.57 for *p,p'*-DDD. It appears that large quantities of HCHs, DDXs, and PAHs occurring as bound residues in the soil were mobilized during gastrointestinal extraction. It should be pointed out that the bound residue may not be completely mobilized and further study using ^{14}C labeled compounds as tracers is recommended to investigate the fate of bound residue in the gastrointestinal model. It appears that the results of organic solvent extractions should be interpreted with caution in bioaccessibility evaluation. Given similarity in properties and behaviors, it is expected that the bound residue of other HOCs may also be mobilized in the gastrointestinal model. It is well recognized that as an aging phenomenon, sequestration is a time-dependent process, and the amounts of sequestered pollutants increase over time (*29, 30*). The soils collected in this study had been contaminated for decades, therefore, the R_b values are likely to be higher than those of freshly contaminated soils.

Effects of OC Content and Contaminant Concentration. The mean R_b values of HCHs, DDXs, and PAHs were 0.20, 0.20, and 0.14 for soil A, 0.46, 0.37, and 0.36 for soil B, and 0.51, 0.47, and 0.47 for soil C, respectively. As shown in Figure 2, a positive correlation between OC content and R_b for total concentrations of HCHs (ΣHCHs), DDXs (ΣDDXs), and PAHs (ΣPAHs) is demonstrated. Such a correlation was also true for most individual compounds studied. The relationships were not tested statistically because of the small sample size (3 soils only). In addition, R_b can also be affected by other factors including properties of chemicals and composition of soil organic matter. The correlation shown in Figure 2 reflects no more than a trend, rather than quantitative relationship. Still, there was very likely a trend that R_b values

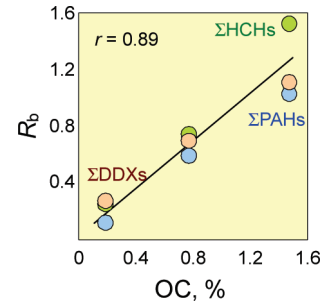

FIGURE 2. Relationships between the extracted bound ratio (R_b) and OC (left panel) and between the quantity of bound residue (Q_{bound}) and the quantity of unbound residue ($Q_{unbound}$) (right panel).

of a soil with higher OC content are generally higher than those of a soil with lower OC content, given that the total concentrations of contaminants and OC compositions are similar. Significant correlation between the undegradability of phenanthrene and OC content was also reported in the literature (*31, 32*). Soils with higher OC contents often have higher potential for sequestrating HOCs than those with lower OC contents because HOCs are mainly trapped in OC (*33, 34*). It is likely that OC of the soils studied had trapped and accumulated contaminants over a prolonged period of pollution and the higher the OC content, the more the bound residue accumulated.

Role of Bile Salt. The previously presented results were derived from an experiment using a typical gastrointestinal model with simulated gastric (pepsin), intestinal (pancreatin, lipase, bile salt), and α-amylase digestive fluids. To identify the key components responsible for mobilization of the bound residue, the soil B was extracted using different combinations of these constitutes including one without any enzyme, and the results for ΣHCHs, ΣDDXs, and ΣPAHs are presented in Figure 3. A one-way ANOVA with multiple comparison was conducted to test the differences in the extracted total quantities among the different combinations of digestive compositions. For all the three types of contaminants studied, the extracted quantities with gastrointestinal digestion were significantly higher ($p < 0.05$) than those without, indicating the apparent mobilization of the bound residues during digestion. Moreover, there was no significant difference ($p > 0.05$) among the five combinations of digestive constitutes, indicating that bile salt alone can mobilize the bound residues, and the extractability was not enhanced by introducing the other constituents.

Pepsin, pancreatin, lipase, and α-amylase in the digestive solution can break down fat, protein, and carbohydrates, respectively (*35*). Apparently, they were not active in mobilizing the bound residues of HOCs in the soil. The role of bile salt in the gastrointestinal digestion has been extensively investigated. It was demonstrated that bile salt

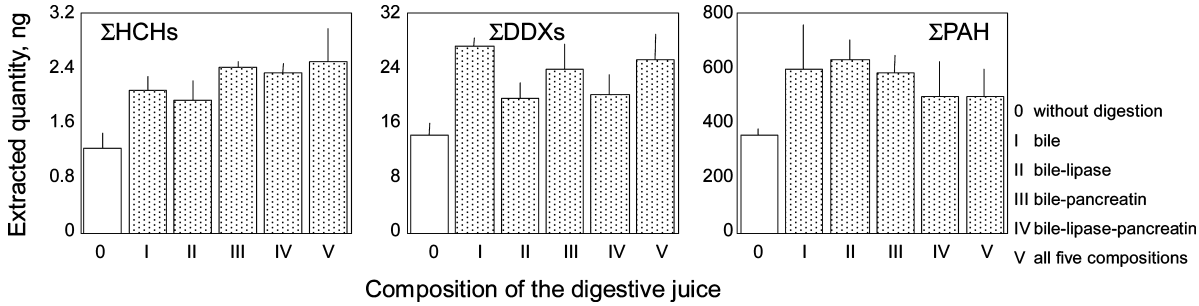

FIGURE 3. Effect of compositions of the digestive solution on the total quantities of ΣHCHs, ΣDDXs, and ΣPAHs extracted from soil B (shaded bars) in comparison with a control (blank bars) without the digestion. Means and standard deviation are presented.

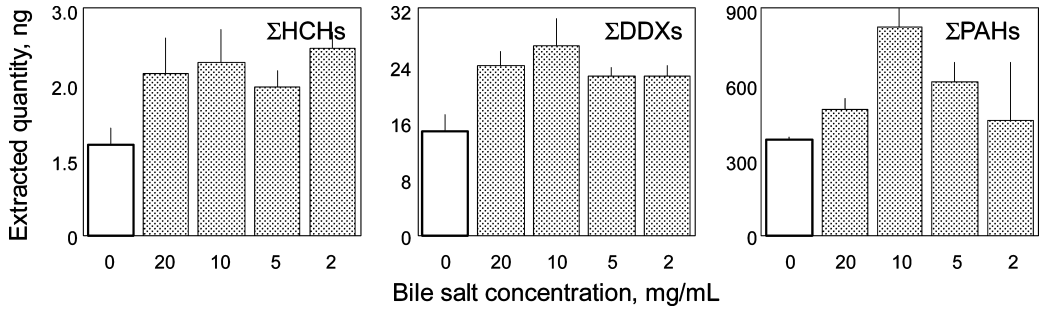

FIGURE 4. Effect of bile salt concentration on the quantities of ΣHCHs, ΣDDXs, and ΣPAHs extracted from soil B in comparison with a control without digestion (labeled as 0 bile salt concentration). Means and standard deviations are presented.

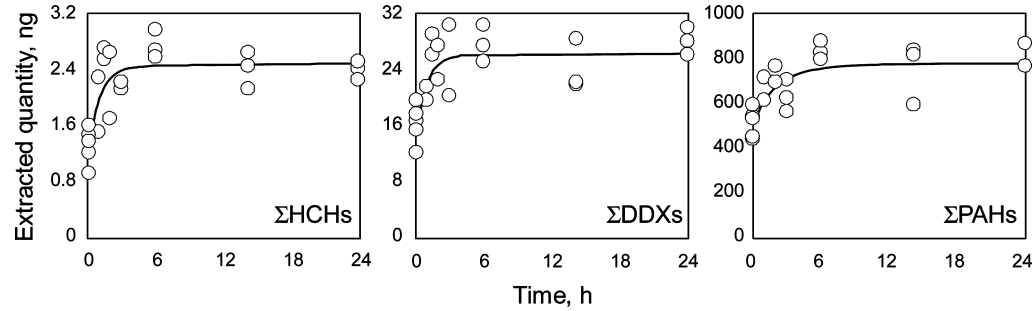

FIGURE 5. Mobilization kinetics of bound residue of ΣHCHs, ΣDDXs, and ΣPAHs from soil B by bile salt of 10 mg/L.

forms mixed micelles with fatty acids and other compounds such as lecithin in gastrointestinal tract (*36*), and a large fraction of the mobilized HOCs can be incorporated into the apolar interior of the micelles (*36–38*). Most studies on gastrointestinal bile salt focused on the central role of the micelles for retaining contaminants in the fluid and facilitating the transport of them across the boundary layer on the surface of the intestinal wall (*36–39*). It was also found that bile salt in the aqueous phase initially enhanced PAH desorption from soil and the desorbed PAHs formed large aggregates with bile, reducing the freely dissolved PAHs in the supernatant (*14*). In addition to reducing the quantities of the mobilized chemicals freely dissolved in digestive fluid, the interaction between the bile salt and soil matrix cannot be ruled out. It was shown that the addition of bile salt to artificial digestive juice increased the amount of PAHs and PCBs released in an in vitro model significantly and a surfactant-like mechanism for HOC mobilization was proposed (*40*). Still, the mobilization process was not fully understood (*14*) and further study on mobilization mechanism is recommended.

Effect of Bile Salt Concentration. Knowing that bile salt was the only active constitute in gastrointestinal digestive juice which mobilized bound residues of OCPs and PAHs in contaminated soil, the influence of bile salt concentration on extractability was studied using soil B and the results are shown in Figure 4. Again, in addition to various bile salt concentrations, a set of experiments with no bile salt digestion was conducted for comparison (concentration 0 in Figure 4). The results of a one-way ANOVA and multiple comparison revealed that digestion increased the extractability significantly ($p < 0.05$), while the total extracted quantities of ΣHCHs, ΣDDXs, and ΣPAHs were not affected by bile salt concentration within the investigated range from 2.0 to 20 mg/mL. It appears that bile salt was critical in mobilizing the bound residue of the tested contaminants from the soil, while 2 mg/mL of bile salt was enough for such a mobilization.

Mobilization Kinetics. Figure 5 illustrates mobilization kinetics of bound residue of ΣHCHs, ΣDDXs, and ΣPAHs from contaminated soil B which was digested with bile salt of 10 mg/L for various time periods from 0 to 24 h before extraction for HCHs, DDXs, and PAHs. The directly extracted quantities without digestion are shown as time 0. It appears that the mobilized quantities of bound residue increased quickly by digestion during a relatively short period of time around 3 h.

It appears that the time trends of the mobilized bound residue quantities followed a typical first-order kinetics. Therefore, a single exponential rise to maximum function was used to fit the data. With positive values at origin due to nonbound residue (extracted without digestion), a three-, instead of a two-parameter exponential function with an interception was applied. The following equations were derived based on the least-squares regression:

$$Q_{bound} + Q_{unbound}(HCHs) = 1.22 + 1.086(1 - e^{-0.995t}), \quad r^2 = 0.611$$

$$Q_{bound} + Q_{unbound}(DDXs) = 15.0 + 9.68(1 - e^{-1.036t}), \quad r^2 = 0.694, \text{ and}$$

$$Q_{bound} + Q_{unbound}(PAHs) = 356 + 187(1 - e^{-0.474t}), \quad r^2 = 0.641$$

where $Q_{bound} + Q_{unbound}$ (HCHs), $Q_{bound} + Q_{unbound}$ (DDXs), and $Q_{bound} + Q_{unbound}$ (PAHs) are the total quantities of ΣHCHs, ΣDDXs, and ΣPAHs (ng/g) extracted after digestion, respectively, and t represents the digestion time (h). When t equals to zero, the calculated values are the directly extracted HOC quantities without digestion. When t approaches infinite, $Q_{bound} + Q_{unbound}$ (HCHs), $Q_{bound} + Q_{unbound}$ (DDXs), and $Q_{bound} + Q_{unbound}$ (PAHs) equal the extracted total quantities of both nonbound and bound residue fractions. Therefore, the second parameter of each equation represents the quantity of bound residue that was mobilized by digestion. For ΣHCHs, ΣDDXs, and ΣPAHs in soil B, the extracted bound residues were 1.04, 9.37, and 206 ng, accounting for 46, 37, and 36% (R_b) of the total, respectively. Finally, the rate constants (the constants in the exponential term) represent how fast the bound residues of HOCs could be mobilized. The rate constants of ΣHCHs and ΣDDXs were similar to each other and 90% of the bound residue of the two OCPs was mobilized within 2.4 h, while the rate constant of ΣPAHs was approximately half of those for OCPs, and 4.8 h was required for 90% of bound ΣPAHs residue to be mobilized.

Discussion

It was demonstrated by both laboratory and field experiments that fugacities of nonmetabolizable HOCs in the gastrointestinal tract of fish were 7−8 fold higher than those in the consumed food and the increase in fugacities is the primary mechanism of biomagnification (*41, 42*). It was also found that the elevation of fugacities was mainly due to food digestion in the gastrointestinal tract and food absorption from the gastrointestinal tract. If a significant amount of bound residue of HOCs in food can be mobilized in the gastrointestinal tract as shown in this study, the release of the bound residue can be another reason leading to elevated chemical fugacities in the gastrointestinal tract.

For risk assessment on unintentional ingestion of contaminated soil, possible mobilization of bound residue should be taken into consideration. The result of this study implies that if the total concentrations measured using conventional solvent extraction procedure were applied without taking into consideration the sequestered fraction, which is usually not extractable using conventional solvent extraction procedure, the exposure risk could be underestimated even assuming that all extractable chemicals ingested can be absorbed. It is also very likely that the application of chemical equilibrium model for oral exposure assessment of chemicals, particularly poorly metabolizable substances, in soil can underestimate the risk. Only contaminated soils were investigated in this study. In fact, bound residue of HOCs also occurs in other media including various foods (*43*). It will be interesting indeed to investigate possible remobilization of bound residues of various HOCs in food in the gastrointestinal tract.

The conclusion derived from this study was based on comparisons in extracted quantities between the procedures with and without gastrointestinal digestion. In future studies, comparison experiments using pre-extracted soil samples with only bound residue left can provide better evidence for testing this hypothesis.

Acknowledgments

The funding for this study was provided by National Basic Research Program (2007CB407301), the Ministry of Environmental Protection (200809101), and the National Scientific Foundation of China (40730737) and NIEHS (P42 ES016465). We thank Eva Land (Georgia Institute of Technology) for the language improvement of the manuscript.

Supporting Information Available

Preliminary experiments on the effectiveness of individual constituents. These materials are available free of charge via the Internet at http://pubs.acs.org.

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ES1025849