Environmental Science & Technology

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Intrinsic Clearance of Xenobiotic Chemicals by Liver Microsomes: Assessment of Trophic Magnification Potentials

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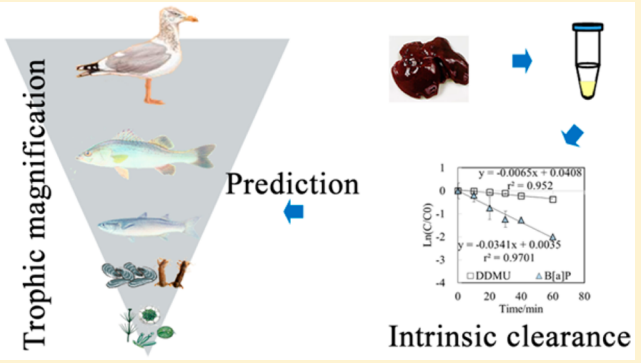
Supporting Information

ABSTRACT: The use of trophic magnification factors (TMFs) to characterize the bioaccumulation potentials of chemicals was encouraged; however, the method for the assessment of trophic magnification potentials is still lacking. We optimized the in vitro assays used for the measurement of intrinsic clearance in liver microsomes by incorporating benzo[a]pyrene (B(a)P) as a benchmark compound. The intrinsic clearance of 40 compounds was then measured in microsomes from fish (weevers) and birds (quail); the characteristics of the trophic transfer of these 40 compounds were previously investigated in an aquatic food web in Bohai in northern China. Chemicals that are biotransformed at a rate similar to or higher than that of B[a]P in the microsomes of both weevers and quail (in vitro intrinsic clearance values, CL; $CL/CL_{B[a]P}$: 0.1 to 2.4) generally exhibited no significant trophic magnification or dilution in the food web (TMF ≈ 1 or < 1), whereas chemicals that are biotransformed at extremely slow rates compared with B[a]P ($CL/CL_{B[a]P}$: 0 to 0.2) showed significant trophic magnification in the food web (TMF > 1). The in vitro intrinsic clearance values of the target chemicals were found to be consistent with their respective trophic transfer behavior in the aquatic food web. Significant negative correlations were also found between the TMFs and the intrinsic clearance values of all target chemicals obtained in microsomes from both weevers and quail. Multiple linear regression analysis showed that biotransformation rates ($CL/CL_{B[a]P}$) are a more important factor compared with the lipophilicity of the chemicals (log K_{ow}) in the assessment of the trophic magnification of chemicals in the aquatic food web.



INTRODUCTION

The persistent organic pollutants (POPs) protocol endorsed by more than 100 countries in 2004 has led to significant activities in the assessment of persistent, bioaccumulative, and toxic substances throughout the world.[1] Bioaccumulation is a fundamental process in environmental toxicology and risk assessment because it determines the internal dose of environmental pollutants.[2] Bioaccumulation potentials have been routinely scrutinized by regulatory agencies across the globe as part of the risk assessment of chemicals.[3] Governments worldwide have sought to evaluate all commercial chemicals to identify substances that undergo trophic magnification in the food web and achieve harmful concentrations in organisms.[4]

The screening and assessment of bioaccumulation generally rely on laboratory-derived bioconcentration factors (BCFs) or field-determined bioaccumulation factors (BAFs).[5] In the past few decades, studies of pollutants in food webs have demonstrated that bioaccumulation in food webs is not solely a lipid−water partitioning process. During a global "Lab-Field Bioaccumulation Workshop" held in New Orleans, Louisiana, the use of trophic magnification factors (TMFs) to characterize the bioaccumulation potentials of chemicals was encouraged because they provide a characterization of the average degree of trophic magnification that occurs across an entire food web.[4,6−8] The hydrophilicity (water solubility) of compounds is considered to be an important determinant of the trophic magnification of chemicals in aquatic environments. A good correlation between TMFs and the lipophilicity of the chemicals (log K_{ow}) has been reported for recalcitrant polychlorinated biphenyls (PCBs), chlorobenzenes (ClBs), chlordanes, hexachlorocyclohexanes (HCHs), dichlorodiphenyltrichloroethane (DDTs), mirex, and dieldrin in the North Water Polynya marine food web.[9] However, most trophodynamic studies have focused on POPs or emerging POPs, which generally showed trophic magnification in aquatic food webs. Some studies also found that compounds with a similar log K_{ow} value did not biomagnify (e.g., 4-nonylphenol [NPs] or phthalate esters [PEs]) or even exhibited trophic dilution (e.g., polycyclic aromatic hydrocarbons [PAHs] or hydroxylated polybrominated diphenyl ethers [OH-PBDEs]) in aquatic food webs, which suggests that other factors also affect TMFs.[10−13] Biotransformation has long been recognized as an important source of uncertainty in predictions of

Received: March 8, 2016
Revised: April 28, 2016
Accepted: May 6, 2016
Published: May 6, 2016

DOI: 10.1021/acs.est.6b01178
Environ. Sci. Technol. 2016, 50, 6343−6353

bioaccumulation,[14,15] and biodegradation testing is required for bioaccumulation assessments under the Chemical Substance Control Law in Japan.[3] However, the influence of biotransformation on the trophic transfer of chemicals is unknown due to the limited availability of biological biotransformation rates and their relationships with reported TMFs.

Currently, the methods most often adopted for accurately and efficiently determination of biotransformation rates are still lacking. Among the various in vitro methods, subcellular fractions of the liver (e.g., microsomes or S9) have been shown to be best suited to the high-throughput screening of chemicals' biotransformation potentials.[14,16] A recent study demonstrated that cryopreserved trout hepatocytes can be used to reliably obtain in vitro intrinsic clearance of xenobiotics.[17] The results of that study also suggested that the incorporation of one or more "benchmarking" chemicals into in vitro chemical depletion could potentially reduce the variation between different batches.[17] However, information regarding the nature of an ideal benchmarking compound (e.g., the desired pathway for metabolism and ease of analysis) is still lacking.

Bohai is an enclosed inland sea in northern China. In recent decades, the trophodynamics of about 40 chemicals have been studied in the Bohai aquatic food web, which includes phytoplankton and seston, zooplankton, invertebrates, fish, and birds.[10,12,13,18−21] The resulting TMFs database of the 40 chemicals obtained from the same aquatic food web provides an excellent opportunity to examine the influence of biotransformation and log K_{ow} values on the trophic magnification of various chemicals. In this study, microsomes of the marine weever (*Lateolabras japonicus*) and common quail (*Coturnix coturnix*) were used to obtain the intrinsic biotransformation rates. The weever is a typical predatory fish that has a large body size and a high trophic level (3.88 ± 0.49) in the Bohai aquatic food web.[10,19] Quail were used to replace seagulls because fresh seagull liver tissue could not be obtained. An in vitro method for the determination of repeatable biotransformation rates was developed and applied to compounds with field-derived TMFs values. The relationships between the log K_{ow} values, biotransformation rates, and TMFs were explored to establish a method to predict the trophic magnification of chemicals in aquatic food webs.

MATERIALS AND METHODS

Chemicals and Reagents. A total of 13 individual PAHs standards (acenaphthene [ACE], fluorene [FE], phenanthrene [PH], anthracene [AN], fluoranthene [FL], pyrene [PY], chrysene [CH], benzo[k]fluoranthene [B(k)F], benzo[a]pyrene [B(a)P], benzo[*ghi*]perylene [BP], dibenz[*a,h*]anthracene [DA], benzo[b]fluoranthene [B(b)F], and benz[a]anthracene [B(a)A]) and four surrogate standards (acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{14}) were obtained from AccuStandard (New Haven, CT). A total of eight individual PBDEs (BDE28, BDE47, BDE99, BDE100, BDE119, BDE153, BDE154, and BDE183), mirex, seven individual PCBs (PCB105, PCB114, PCB118, PCB123, PCB157, PCB169, and PCB189), and ^{13}C-labeled PCBs were obtained from Wellington Laboratories Inc. (Guelph, ON, Canada). 2′-OH-6′-Cl-BDE7, 6-OH-BDE47, 6′-MeO-BDE17, and 6-MeO-BDE47 were synthesized in the Department of Biology and Chemistry, City University of Hong Kong. HCB and *p,p*′-DDE were purchased from Chemservice (Chester, England), *p,p*′-DDMU was obtained from Sigma (St. Louis, MO), and their surrogates (PCB121 and PCB 198) were obtained from AccuStandard. 4-NP (technical grade) and 4-*n*-NP were purchased from Kanto Chemicals (Tokyo, Japan). NPEOs was purchased from Hayashi Pure Chemicals (Tokyo, Japan). Monobutyltin trichloride (MBT) was purchased from Acros Organics (Geel, Belgium), dibutyltin dichloride (DBT), tributyltin chloride (TBT), and triphenyltin chloride (TPT) were purchased from Wako (Osaka, Japan). Deuterated organotins (MBT-d_9, DBT-d_{18}, TBT-d_{27}, and TPT-d_{15}) were obtained from Hayashi Pure Chemicals. Dichloromethane (DCM), *n*-hexane, acetone, acetonitrile, and methanol of pesticide residue grade were obtained from Fisher Chemicals (Fair Lawn, NJ). Methyl chloroformate was obtained from Sigma-Aldrich. *O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) was obtained from Supelco (Bellefonte, PA). Sodium tetraethylborate ($NaBEt_4$) was purchased from Wako. Water was prepared with a Milli-Q Synthesis water purification system (Millipore, Bedford, MA). A nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system and dithiothreitol (DTT) were purchased from Promega (Madison, WI). Sodium sulfate (analytical reagent grade, Beijing Chemicals) was baked at 450 °C for 4 h and stored in a drying oven before use. Monopotassium phosphate, dipotassium phosphate, ethylene diamine tetraacetic acid (EDTA), glycerol, and sucrose were purchased from Beijing Chemicals.

In Vitro Microsomal Incubations. B[a]P was added to the incubation mixtures as a benchmark compound to normalize the variation in the transformation rates that occurred in different batches of analyses. The details of the preparation of microsomes are provided in the Supporting Information. The protein concentrations were determined with the Bradford method using bovine serum albumin as a standard and according to the manufacturer's protocol (Sigma-Aldrich). Hepatic microsomal CYP450 enzymes and four specific CYP enzymes (7-ethoxyresorufin O-deethylase [EROD], 7-pentoxyresorufin O-demethylase [PROD], 7-methoxyresorufin O-demethylase [MROD], and 7-benzyloxyresorufin O-dealkylatase [BROD]) from weevers and quail were measured with fluorescence kits (Genmed Scientific Inc.). The EROD, PROD, MROD, and BROD were markers for CYP1A1, CYP2B1, CYP1A2, and CYP3A1 activity, respectively.

For in vitro incubations of weever microsomes, the reactions were performed in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT, and 20% (v/v) glycerol. The reaction mixtures (200 μL) consisting of 100 μL of liver microsomes (2 mg/mL protein), 60 μL of NADPH regenerating system, and 1 μL of substrate (0.5 μM for both individual compounds and B[a]P) and were incubated in 1.5 mL amber glass vials. Reactions were initiated by adding a NADPH-generating system (NADP, 6.5 mM; glucose 6-phosphate, 16.5 mM; $MgCl_2$, 16.5 mM; and glucose 6-phosphate dehydrogenase, 2 U/mL). Incubations were carried out in triplicate in separate glass vials at 25 °C, and 200 μL of ice-cold acetone was added into each vial to terminate the reaction after 0, 1, 3, 5, 9, 12, and 24 h.

For in vitro incubations of quail microsomes, 50 mM phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$ and 0.5 mM EDTA was added into the incubation vials. The reaction mixtures (200 μL) consisted of 100 μL of liver microsomes (2 mg/mL protein), 60 μL of NADPH regenerating system, and 1 μL of substrate (0.5 μM for individual compounds and 2 μM for B[a]P) and were incubated in 1.5 mL amber glass vials. Reactions were initiated by adding a NADPH-generating system (NADP, 6.5 mM; glucose 6-phosphate, 16.5 mM;

DOI: 10.1021/acs.est.6b01178
Environ. Sci. Technol. 2016, 50, 6343−6353

Table 1. Activities of Hepatic Microsomal CYP450 Enzymes and Four Specific CYP Enzymes (EROD, PROD, MROD, and BROD) from Weevers and Quail and Range Concentrations of Enzyme Activities in Hepatic Microsomes of Marine Fishes and Birds from References

	weevers	marine fishes in ref[a]	quail	birds in ref[b]
enzyme activity (pmol/mg/min)	mean ± SD	range	mean ± SD	range
total CYP450 enzymes	101 ± 46	43−230	300 ± 68	NV
specific CYP enzymes				
EROD	3.0 ± 0.3	2−10	6.8 ± 0.1	2.2−60
PROD	0.1 ± 0.01	0.2−1	1.7 ± 0.8	0.9−2.6
MROD	3.2 ± 0.5	3.6	10.5 ± 2.2	1.7−32.8
BROD	2.4 ± 0.3	3.7	10.7 ± 0.3	11

[a]Ref from 22−27. [b]Ref from 28−34, NV: no values reported.

$MgCl_2$, 16.5 mM; and glucose 6-phosphate dehydrogenase, 2 U/mL). Incubations were carried out in triplicate at 39 °C in separate glass vials, and 200 μL of ice-cold acetone was added into each vial to terminate the reaction after 0, 10, 20, 30, 40, and 60 min

The vials were stored at −20 °C until chemical analysis. The microsomes were inactivated by boiling for 10 min and were then cooled on ice. Incubations with deactivated microsomes and standards were used as negative controls to assess potential background interference and the possibility of nonenzymatic changes. The percentage variations of concentrations of target compounds along with the incubation time in the deactivated liver microsomes were shown in Figures S1 and S2.

Sample Analysis. The incubation mixture in each vial was added with 1 mL of water and surrogates and extracted three times with 1 mL of hexane. The aquatic fraction was then passed through a Pasteur pipe filled with sodium sulfate to remove moisture and eluted with 1 mL of hexane and 1 mL of DCM, which were also used to rinse the glass vial before elution. All of the extracts were combined and concentrated to 50 μL for instrumental analysis. The organotins were derivatized with $NaBEt_4$ before gas chromatography−electron ionization−mass spectrometry (GC-EI-MS) analysis, 4-NP was derivatized with BSTFA before GC-EI-MS analysis, and 6-OH-BDE47 was derivatized with methyl chloroformate before gas chromatography−negative chemical ionization−mass spectrometry (GC-NCI-MS) analysis. PAHs, PCBs, DDTs, HCB, mirex, organotins, and 4-NP were directly analyzed by GC-EI-MS (Agilent Technologies); PBDEs, 6-MeO-BDE47 and 6-OH-BDE47 were analyzed by GC-NCI-MS (Shimadzu QP 2010 plus); and NPEOs were analyzed with high-performance liquid chromatograph (Agilent 1100 Series) coupled with a fluorescence detector (Agilent 1200 Series). Detailed information on the instrumental conditions for each group of chemicals is provided in the Supporting Information.

Quality Assurance and Quality Control. Concentrations of PAHs in sample extracts were quantified relative to deuterated PAHs (chrysene-d_{12}, perylene-d_{14}, and perylene-d_{14}); PBDEs and 6-MeO-BDE47 were quantified relative to 6′-MeO-BDE17; 6-OH-BDE47 was quantified relative to 2′-OH-6′-Cl-BDE7; *p,p*′-DDE, *p,p*′-DDMU, and HCB were quantified relative to PCB 121; 4-NP was quantified relative to 4-*n*-NP; organotins were quantified relative to deuterated organotins (MBT-d_9, DBT-d_{18}, TBT-d_{27}, and TPT-d_{15}); mirex was quantified to PCB 198; and PCBs were quantified relative to ^{13}C-labeled PCBs. The recoveries of surrogates standards were 78 ± 19%, 100 ± 1%, 85 ± 13%, 85 ± 6%, 72 ± 19%, 124 ± 7%, 93 ± 26%, and 119 ± 14% for deuterated PAHs, 6′-MeO-BDE17, PCB 121, PCB198, 2′-OH-6′-Cl-BDE7, 4-*n*-NP, deuterated organotins, and ^{13}C-labeled PCBs, respectively.

Data Analysis. The declining concentrations of substrate in the incubation mixtures over time followed the monoexponential decay model and were fit to the linear eq 1:

$$\ln C_t = \ln C_0 - K \cdot t \quad (1)$$

where t is incubation time; C_0 and C_t are the substrate concentrations in the incubation medium at time zero and time t, respectively; and K is the apparent first-order biotransformation rate constant (h^{-1}). The in vitro intrinsic clearance values (CL, mL/h/mg of protein) were obtained according to the eq 2:

$$\mathrm{CL} = \frac{K}{C_{\mathrm{protein}}} \quad (2)$$

where C_{protein} is the protein concentration (mg protein/ml) of the incubation mixtures. The in vitro intrinsic clearance value of each chemical was normalized to that of B[a]P according to the eq 3:

$$\mathrm{CL}' = \frac{\mathrm{CL}}{\mathrm{CL}_{\mathrm{B[a]P}}} \quad (3)$$

where CL′ is the normalized intrinsic clearance value of target compounds, and $\mathrm{CL}_{\mathrm{B[a]P}}$ is the intrinsic clearance value of B[a]P.

Statistical Analysis. Multiple linear regression models were constructed to determine the contribution of log K_{ow} and the biotransformation rate on the trophic magnification of chemicals. TMFs were slopes of the correlations between concentrations of chemicals in organisms and trophic levels. The significances of the correlations were examined by Spearman's rank correlation test. The TMFs were log10-transformed before analysis, and the linear regression was regarded as significant when the p value was less than 0.05. The correlations between $\mathrm{Ln}(C_t/C_0)$ and the incubation time were also examined with Spearman's rank correlation test. The linear regression was regarded as significant when the value of p was below 0.05. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL).

RESULTS AND DISCUSSION

Hepatic Microsomal Enzyme Activities. The activities of hepatic microsomal CYP450 enzymes and the four specific CYP enzymes (EROD, PROD, MROD, and BROD) in weevers and quail are listed in Table 1. The total and specific CYP450 enzyme activities in the microsomes of weevers were within the ranges of those in marine fish reported

DOI: 10.1021/acs.est.6b01178
Environ. Sci. Technol. 2016, 50, 6343−6353

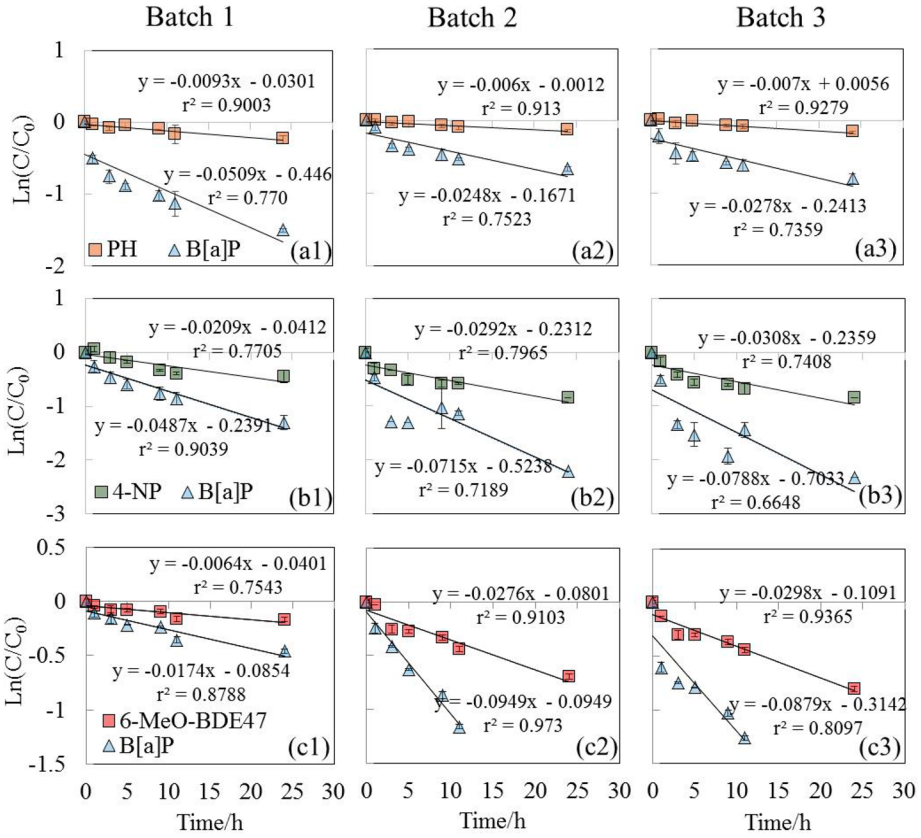


Figure 1. Substrate depletion curves for the three test chemicals (PH [a1−a3], 6-MeO-BDE47 [b1−b3] and 4-NP [c1−c3]) with B[a]P as the benchmark compound in weever microsomes. Each graph represents the data from one batch for one test chemical. The error bars indicate the standard deviation of the triplicate runs. CYP450 enzyme activities were 103, 168, and 171 (pmol/mg/min) for batches 1, 2, 3, respectively. Concentrations of B[a]P at 24 h for C2 and C3 were below the detection limits. Concentrations of C_0 were in the range of 0.49−0.54 μM.

previously,[22,23] and the profiles of EROD, MROD, PROD, and BROD activities were also similar to those of the corresponding enzymes in previous investigations.[24−27] In the quail liver microsomes, the EROD, MROD, PROD, and BROD activities were determined to be 6.8 ± 0.1, 1.7 ± 0.8, 10.5 ± 2.2, and 10.7 ± 0.3 pmol/mg/min, respectively, which were all within the normal ranges reported previously for herring gulls, glaucous gulls, yellow legged gulls, chickens, black-legged kittiwake, European quail, gray partridges, and northern fulmar.[28−34] Similar hepatic enzyme activities observed between quail and other avian species suggested that quail could be used as an acceptable model to replace seagulls in this study. The incubation of chemicals with quail microsomes was conducted for 1 h due to the relatively high activities of hepatic microsomal CYP enzymes, and incubations of chemicals with weever microsomes were extended to 24 h to evaluate biotransformation potentials of persistent compounds (e.g., PCBs). A similar incubation time (24 h) was also applied in the incubation of fish microsomes in previous studies.[35−37] The enzyme activities of EROD, PROD, MROD, and BROD, along with the incubation time, were determined in microsomes of weevers and quail, and no significant decline was observed for the target enzyme activities (Figure S3), which suggests that biotransformation can continue for up to 24 h during in vitro incubations.

Method Optimization. The reliability of the in vitro assays for assessment of bioaccumulation is substantially hampered by the high variability in enzyme viability and metabolic capacity between different batches. This variability might be accounted for with the use of benchmark chemicals with known metabolic characteristics.[14,17] In this study, B[a]P was tested as a benchmark compound in in vitro assays to normalize the variation that occurred across different batches of analyses. B[a]P was reported to undergo biotransformation mainly through CYP1A1,[38] and the depletion of the chemical followed first-order kinetics with a relatively constant first-order rate constant in previous studies.[15−17,39,40] A total of three chemicals (PH, 6-MeO-BDE47, and 4-NP) from diverse chemical classes were used to assess the variability in the assays because PH, 6-MeO-BDE47, and 4-NP undergo steadily biotransformation by CYP1A1, CYP2B, and CYP1A2, respectively.[41−43] The activities of EROD, PROD, and MROD (markers for CYP1A1, CYP2B1, and CYP1A2, respectively) have been determined to be within normal ranges in microsomes of weevers (Table 1). Incubation of B[a]P with chemicals biotransformed by the same or different metabolic enzymes in microsomes could help to clarify whether the benchmark chemical would reduce the variations within different enzymes. Each chemical was tested relative to B[a]P in the microsomes of weevers in triplicate batches at a low substrate concentration (0.5 μM). The low substrate concentration prevented enzyme saturation and high cytotoxicities of the chemicals and allowed for the detection of significant depletion of substrates. The microsomes for each batch were isolated at different times (January, July, and November 2014). As shown in Figures 1−3, the in vitro intrinsic clearance values of PH, 6-MeO-BDE47, and 4-NP were 0.007 ± 0.0017, 0.027 ± 0.005, and 0.021 ± 0.013 (mL/h/mg of protein) with the variation (% CV) in

DOI: 10.1021/acs.est.6b01178
Environ. Sci. Technol. 2016, 50, 6343−6353

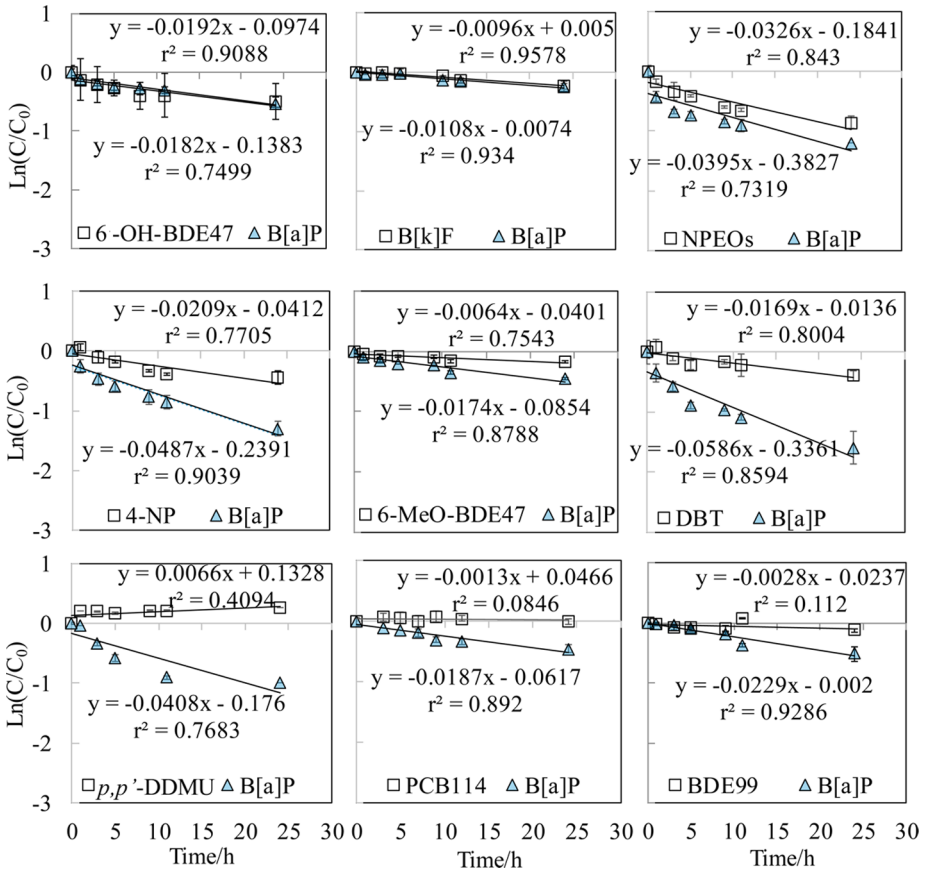


Figure 2. Substrate depletion curves for chemicals that undergo trophic dilution, as exemplified by 6-OH-BDE47, B[k]F, and NPEOs ($CL/CL_{B[a]P}$: 0.14−1.0); chemicals that show no trophic magnification, as exemplified by 4-NP, 6-MeO-BDE47, and DBT ($CL/CL_{B[a]P}$: 0.10−1.4); and chemicals that exhibit significant trophic magnification, as exemplified by *p,p′*-DDMU, PCB114, and BDE99 ($CL/CL_{B[a]P} \approx 0$) in weever microsomes. Concentrations of C_0 were in the range of 0.43−0.54 μM.

intrinsic clearance values of 23%, 23%, and 61%, respectively. After correction by the intrinsic clearance value of B[a]P, the CL′ values for PH, 6-MeO-BDE47, and 4-NP were 0.22 ± 0.037, 0.41 ± 0.019, and 0.29 ± 0.046, respectively, and the variation (% CV) of the three compounds decreased to 5−17%. The corrected variation was in the range of the reported intralaboratory variability (4.1−30%) achieved when the hepatocyte assay was isolated and cryopreserved in one location.[17] The study's results demonstrate that the incorporation of B[a]P benchmarking could substantially reduce the variability between batches. The variations of enzyme activities caused by cytotoxicity of chemicals and other abiotic factors during the incubations could also be corrected by the depletion of B[a]P in the in vitro system.

Another critical issue that can cause great variation in in vitro systems is the adsorption of chemicals into the reaction vessels or proteins.[14] In this study, a separate amber glass vial (1.5 mL) was used for incubations at different reaction times. This approach differs from previous methods in which an aliquot of the suspension was taken from the reaction vial each time.[17,44,45] Using the amber glass vial helped to avoid the uneven distribution of chemicals in the solutions or adsorption into vessels. The adsorption of chemicals during incubations would also cause substrate loss during liquid−liquid extraction, possibly as a result of the nonspecific binding of chemicals to proteins within the system, a process that could not be reduced by liquid−liquid extraction. An extraction method was developed in this study in which incubation mixtures were passed through a Pasteur pipet filled with anhydrous sodium sulfate, which was then eluted by hexane and DCM. As a result of this method, the target compounds showed 88−114% recovery in all of the incubated samples terminated by ice-cold acetone at 0 h (Figure S4); these values were significantly higher than those in samples in which liquid−liquid extraction was used (40−55%).

Intrinsic Clearances. The substrate depletion approach has been well adopted for estimation of intrinsic clearance for a single compound.[15,17,40,46−48] In this study, the biotransformation of target compounds followed first-order kinetics in both weever and quail liver microsomes (Figures 2 and 3). A summary of the in vitro intrinsic clearance values for the 40 compounds examined are listed in Table 2. Relatively high intrinsic clearance rates were observed for PAHs, 6-OH/MeO-BDE47, NPEOs, 4-NP, and butyltins, and the CL values of these compounds were determined to be 0.0022−0.17 and 0.26−4.6 (mL/h/mg of protein) in the liver microsomes of weevers and quail, respectively. In comparison, no statistically significant decline was found for PBDEs, DDTs, HCB, mirex, PCBs, or TPT during the incubation periods in weever microsomes, and extremely slow depletion was observed for PBDEs and *p,p′*-DDMU in quail microsomes, with a CL of 0.05−0.39 (mL/h/mg of protein). The available substrate depletion studies focused mainly on the hepatic clearance of 4-NP, PAHs, and some pharmaceuticals in fish.[39,46−51] The CL of 4-NP was reported to be 0.18 and 0.11 (mL/h/mg of protein) in trout-liver microsomes and S9 fractions, respectively,[39] which

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Environ. Sci. Technol. 2016, 50, 6343−6353

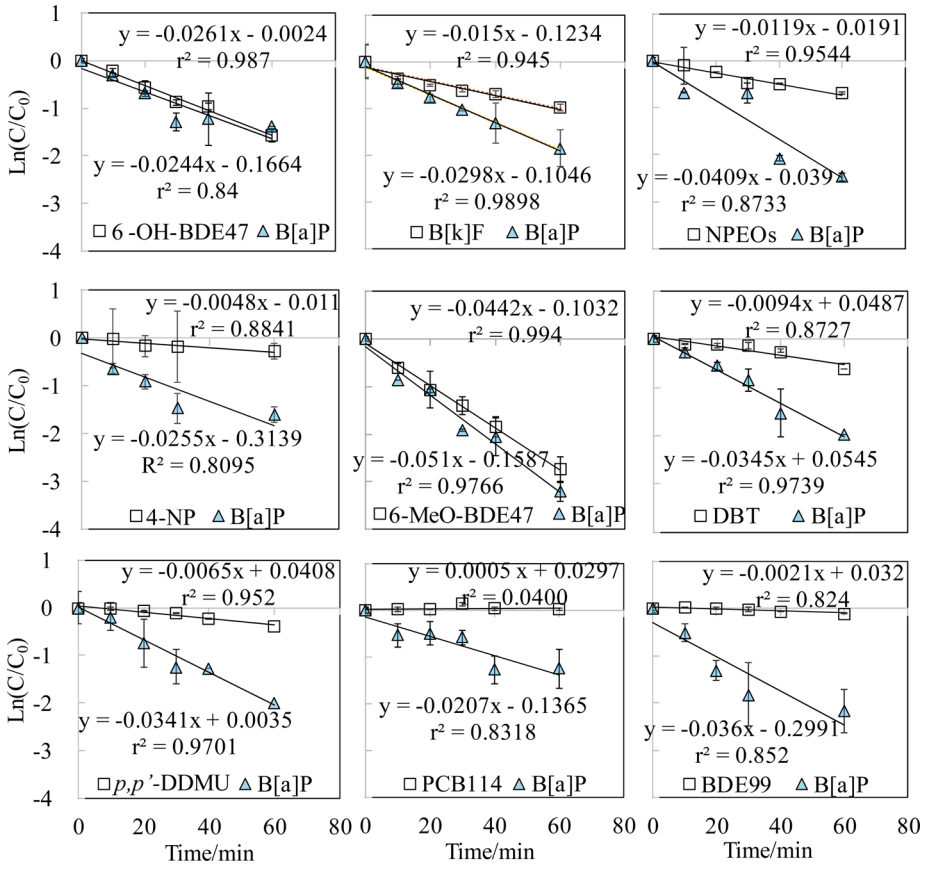

Figure 3. Substrate depletion curves for chemicals that undergo trophic dilution, as exemplified by 6-OH-BDE47, B[k]F, and NPEOs ($CL/CL_{B[a]P}$: 0.5−2.4); chemicals that show no trophic magnification, as exemplified by 4-NP, 6-MeO-BDE47, and DBT ($CL/CL_{B[a]P}$: ≈0−2.6); and chemicals that exhibit significant trophic magnification, as exemplified by *p,p′*-DDMU, PCB114, and BDE99 ($CL/CL_{B[a]P}$: ≈0−0.19) in quail microsomes. Concentrations of C_0 were in the range of 0.5−0.58 μM.

were relatively higher than those found in the weever microsomes in this study (0.021 mL/h/mg of protein). The intrinsic clearance values of B[a]P reported in three separate studies were 0.024 ± 0.003 (mL/h/mg of protein) (dosed concentrations, 0.5 μM),[51] 0.032−0.088 (mL/h/mg of protein) (dosed concentrations, 2 μM),[39] and 0.19−0.37 (mL/h/mg of protein) (dosed concentrations, 1−2.5 μM).[49] The differences between these reported intrinsic clearance rates might have resulted from the use of different trout subspecies, S9 fraction protein concentrations, and beginning substrate concentrations. The CL of B[a]P in weever microsomes (0.03 ± 0.02 mL/h/mg of protein) in this study was in the range of the reported values cited above.

To the best of our knowledge, no information is available about the in vitro intrinsic clearance of chemicals in birds. The extremely low intrinsic clearance values of persistent chemicals (e.g., DDTs, HCB, mirex, PCBs, and TPT) in quail microsomes were similar to the values obtained in weever microsomes (Figures 2 and 3), but the CL_{quail} of the chemicals that undergo steady biotransformation was found to be 14 to 1500 times higher than those in weever microsomes (Table 2). It is interesting to note that the hepatic clearance values of PAHs increased in a linear relationship with benzo-rings in incubations with weever microsomes (Figure 4). The relationship was similar to the increasing degree of stereoselectivity with the size and shape of the molecules in the metabolism of PAHs by bullhead liver microsomes: B[a]P (five benzo-rings) > CH (four benzo-rings) > PH (three benzo-rings).[52−54] However, a negative correlation was observed between the CL'_{quail} of PAHs and their benzo-rings in quail microsomes (Figure 4). The discrepancy between quail and fish for the microsomal metabolism of PAHs was also reported between rats and fish, possibly because of the significantly different stereoselectivities of cytochrome P450 enzymes between the two species.[52−56] The results suggested that quail- and rat-liver microsomal enzymes may have similar stereoselectivity in the metabolism of PAHs.

Assessment of Trophic Magnification Potentials. It has been reported that two important physiological processes determine whether a chemical will bioaccumulate in fish: the uptake of the chemical from the environment and the biotransformation of the chemical into water-soluble metabolites.[14,51] Fish take up chemicals through their gills and intestines, and these pathways depend mainly on the lipophilicity of the chemicals (log K_{ow}).[14,57] Thus, the relationships between field-derived TMFs, log K_{ow}, and in vitro intrinsic clearance values in microsomes from weevers and quail were explored (Table 2). As shown in Figure 5a, TMFs increased with log K_{ow} values for all target compounds, but the correlation between TMFs and log K_{ow} was not statistically significant for the 40 target chemicals in this study (Figure 5a). Although positive correlations have been reported between TMFs and log K_{ow} for some POPs in previous studies,[9,58−60] negative relationships or no relationships were observed for dioxins, PAHs, and PEs.[11,19,61−63] The different relationships

DOI: 10.1021/acs.est.6b01178
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Table 2. Field-Derived TMFs, log K_{ow}, and in Vitro Intrinsic Clearance Values (mL/h/mg of protein) of 40 Target Chemicals

			fish microsomes			bird microsomes		
chemicals	log K_{ow}[a]	TMF[b]	CL	$CL/CL_{B[a]P}$	r^2	CL	$CL/CL_{B[a]P}$	r^2
chemicals that undergo trophic dilution in the aquatic food web								
B[a]P	6.04	0.32	0.03 ± 0.02	1	0.9088	1.8 ± 0.61	1	0.9898
B[k]F	5.8	0.27	0.0096	0.88	0.9578	0.90	0.50	0.9454
PY	5.18	0.17	0.012	0.84	0.9356	2.8	1.6	0.9629
FL	5.22	0.11	0.012	0.81	0.8517	3.4	1.9	0.9257
CH	5.86	0.26	0.010	0.74	0.922	1.8	1.0	0.9745
B[b]F	5.98	0.27	0.016	0.67	0.7945	1.8	1.0	0.9844
AN	4.54	0.34	0.0068	0.28	0.9916	4.2	2.4	0.8843
B[a]A	5.91	0.20	0.0022	0.14	0.9044	2.5	1.4	0.9369
6-OH-BDE47	6.36	0.21	0.018	0.95	0.7499	1.6	1.1	0.987
chemicals that do not exhibit trophic magnification in the aquatic food web								
FE	4.18	1.15	0.0029	0.27	0.8066	4.6	2.6	0.7764
PH	4.57	0.43	0.0029	0.10	0.7702	4.4	2.5	0.7807
ACE	3.92	1.02	0.054	1.1	0.8608	4.0	2.2	0.8473
BP	6.5	0.65	0.0099	0.91	0.965	0.52	0.29	0.8709
DA	6.75	0.85	0.034	1.4	0.7893	0.60	0.34	0.8718
NPEOs	4.72	0.80	0.033	0.83	0.843	0.71	0.29	0.9544
MBT	3	0.77	0.17	0.80	0.9605	NV[c]	NV	NV
4-NP	4.48	0.83	0.021	0.43	0.7705	0.29	0.19	0.8841
DBT	3.12	0.63	0.017	0.29	0.8004	0.56	0.27	0.9557
TBT	4.1	0.59	0.012	0.21	0.8695	0.26	0.12	0.8682
6-MeO-BDE47	6.34	0.55	0.0064	0.37	0.7543	2.7	0.87	0.994
BDE183	8.3	1.59	0.011	0.28	0.7891	NV	NV	NV
BDE28	5.53	1.90	NV	NV	NV	1.1	0.62	0.9374
chemicals that undergo trophic magnification in the aquatic food web								
BDE47	5.95	6.56	NV	NV	NV	0.048	0.01	0.0805
BDE99	6.38	10.02	NV	NV	NV	0.13	0.06	0.8247
BDE100	6.11	7.66	NV	NV	NV	0.096	0.04	0.7807
BDE119	6.44	3.79	NV	NV	NV	0.26	0.12	0.9165
BDE153	7.82	6.12	NV	NV	NV	0.14	0.08	0.7874
BDE154	7.9	5.01	NV	NV	NV	0.13	0.07	0.8217
HCB	5.5	2.95	NV	NV	NV	NV	NV	NV
p,p′-DDE	6.29	3.26	NV	NV	NV	NV	NV	NV
p,p′-DDMU	5.5	3.83	NV	NV	NV	0.39	0.19	0.9523
mirex	6.89	13.00	NV	NV	NV	NV	NV	NV
PCB105	6.65	3.40	NV	NV	NV	NV	NV	NV
PCB114	6.65	9.29	NV	NV	NV	NV	NV	NV
PCB118	6.74	3.40	NV	NV	NV	NV	NV	NV
PCB123	6.74	7.10	NV	NV	NV	NV	NV	NV
PCB157	7.18	3.70	NV	NV	NV	NV	NV	NV
PCB189	7.71	4.64	NV	NV	NV	NV	NV	NV
PCB169	7.42	12.26	NV	NV	NV	NV	NV	NV
TPT	3.5	3.70	NV	NV	NV	NV	NV	NV

[a]Ref from 64−69. [b]Ref from 10, 12, 13, 18−21. [c]NV: no values due to persistence for biotransformation. [d]CYP450 enzyme activities were 84 ± 41 and 309 ± 117 pmol/mg/min for microsomes from weevers and quail, respectively. [e]The underlined TMFs represented statistically significant increases or decreases of the concentrations with trophic levels in aquatic food web ($p < 0.05$). [f]$CL_{B[a]P}$ is listed in Table S1.

between TMFs and log Kow suggested the existence of other, more important factors.

The in vitro intrinsic clearance values of each group of chemicals were found to be consistent with that group's trophic transfer behaviors in the aquatic food web (Table 2). Chemicals that undergo significant trophic dilution in the aquatic food web (TMF < 1, $p < 0.05$), including high-molecular-weight PAHs and OH-PBDEs, were biotransformed at a rapid rate in both weever and quail microsomes ($CL/CL_{B[a]P}$: 0.5−2.4). Biotransformation was difficult for chemicals that showed significant trophic magnification in the food web (TMF > 1, $p < 0.05$), including PBDEs, HCB, DDTs, mirex, and PCBs, in microsomes of both weevers and quail ($CL/CL_{B[a]P}$: 0−0.2). Moderate normalized intrinsic clearance values ($CL/CL_{B[a]P}$: 0.12−1.4) were found for chemicals that exhibited no significant trophic magnification or dilution in the food web (TMF ≈ 1, $p > 0.05$), with the exception of low-molecular-weight PAHs in quail microsomes. Although large species differences in biotransformation were observed within groups of chemicals (e.g., PAHs), similar profiles of intrinsic clearance were found for different groups of chemicals in each species (Table 2). The three groups of chemicals were fractionated by $CL/CL_{B[a]P}$ with the values of 0, 0−0.5 and 0.5−2, respectively, and significant negative correlations were observed between

DOI: 10.1021/acs.est.6b01178
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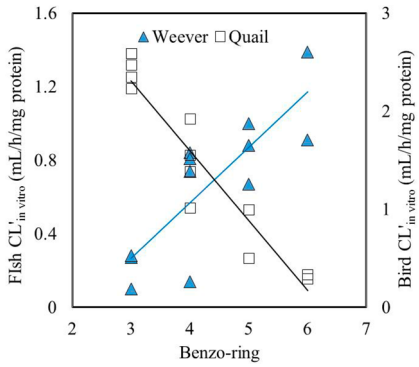

Figure 4. Relationships between number of benzo-rings and in vitro intrinsic clearance values ($CL_{in\ vitro}$) of PAHs in weever and quail microsomes, ▲: CL′ measured in weever microsomes; □: CL′ measured in quail microsomes.

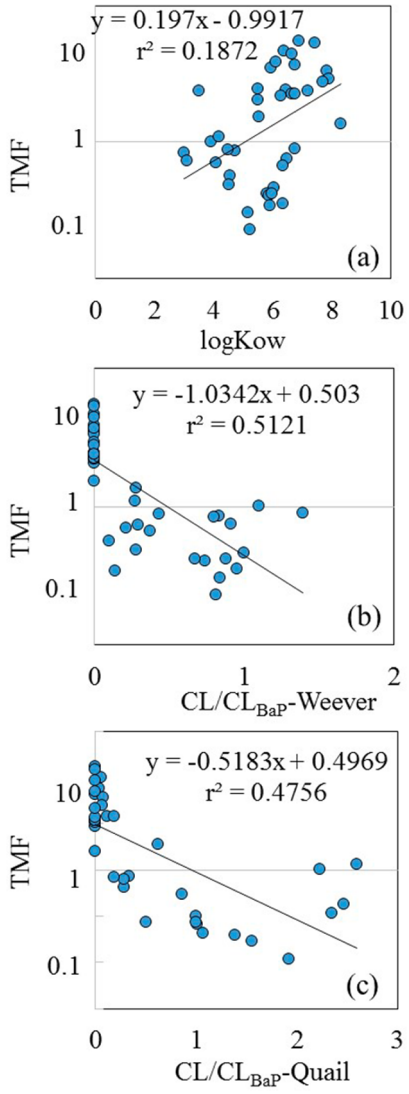

Figure 5. Correlations between field-derived TMFs and log K_{ow} (a) and TMFs and measured biotransformation rates in weever (b) and quail microsomes (c).

TMFs and normalized intrinsic clearance values when the three groups of chemicals were all included in the regression analysis ($p < 0.05$, Figure 5b,c). There are some exceptional chemicals in the correlations (for example, three PAH isomers (FE, PH, and ACE)) had relatively high TMFs in Figure 5c. The possible reason was due to the fact that no significant trophic magnification or dilution was observed for these compounds in the aquatic food web ($p > 0.05$), resulting in the large uncertainties of field-derived TMFs. Multiple linear regression was used to further assess the effects of log K_{ow} and biotransformation on the trophic magnification of chemicals. The analysis produced extremely significant multiple regression models ($p < 0.001$ for all models) (eqs 4 and 5).

$$\log(\mathrm{TMFs}) = 0.119 \log K_{ow}\ (p = 0.024, 6.3\%) - 0.931\ \mathrm{CL'}_{weever}\ (p < 0.001, 51.1\%) - 0.228 \quad (4)$$

$$\log(\mathrm{TMFs}) = 0.092 \log K_{ow}\ (p = 0.137, 3.4\%) - 0.424\mathrm{CL'}_{quail}\ (p < 0.001, 42.3\%) - 0.135 \quad (5)$$

The biotransformation rate was found to be a significant predictor of TMFs, with percentage contributions of 51.1% and 42.3% for weevers and quail, respectively. Multiple linear regressions demonstrated that biotransformation was an important factor in the prediction of the trophic magnification of chemicals in the aquatic food web.

It is well-known that the in vivo models are the most accurate way to determine the clearance rates, but even the abbreviated methods developed recently are expensive and time-consuming.[17] As a result, regulatory agencies throughout the world increasingly favor the development and validation of in vitro methods.[17] Although the use of in vitro system to determine biotransformation rates of chemicals provided a relatively easier and faster way for assessing the trophic magnifications in this study, some toxicokinetic processes such as bioavailability and enterohepatic circulation were not considered in the in vitro system. Because uptake and distribution of chemicals in biotas depend mainly on the lipophilicity of the chemicals (log K_{ow}),[14,57] the biotransformation rates should be applied together with log K_{ow} for assessment of the trophic magnification potentials of chemicals. The relationships between observed and predicted TMF values based on the multiple regression models were shown in Figure S5. Good agreements were obtained for compounds exhibiting significant trophic magnifications or trophic dilutions in the aquatic food web. Most of the outliners in the correlations were compounds that undergo no significant trophic magnification or dilution in the food web, and the calculated TMF values have large uncertainties.

Generally, regulatory authorities rely on the log K_{ow} of a chemical to assess bioaccumulation potentials, and chemicals with log K_{ow} values of greater than 5 are identified as highly bioaccumulative substances. This criterion only reflects the lipid−water partition and neglects biotransformation as another, more important, process for the assessment of bioaccumulation potentials. We optimized the in vitro assays of microsomes and incorporated a benchmark compound into the analysis, which greatly reduced the variability among batches. This method was then applied to 40 compounds whose trophic transfer characteristics were previously investigated in an aquatic food web in Bohai in the northern China. A prediction method for trophic magnification assessment was finally established on the basis of the normalized intrinsic clearance values of all of the target compounds, and a new method was proposed for the identification of chemicals with

DOI: 10.1021/acs.est.6b01178
Environ. Sci. Technol. 2016, 50, 6343−6353

high trophic magnification potentials in an aquatic food web. In addition, only one fish model and one bird model were selected to assess the trophic magnification potentials of chemicals. Further study is needed to explore the variations in the intrinsic clearance values of different animals (e.g., fish) with different trophic levels, which would provide important information about the relationships among TMFs, log K_{ow} values, and biotransformation rates across an entire food web.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01178.

Additional details on microsomal preparations and instrumental analysis of target compounds. Figures showing percentage variations of concentrations of target compounds along with the incubation time in the deactivated liver microsomes of weevers and quail, enzyme activity (EROD, MROD, BROD, and PROD) along with the incubation time in weever liver microsomes (a1-a4) and quail liver micrsomes (b1-b4), recoveries of target compounds in the extraction method developed in the present study, and model predicted versus observed TMFs in various chemicals for weevers and quail. A table showing $CL_{B[a]P}$ values of B[a]P used as benchmark compounds in determining the in vitro intrinsic clearance values of 40 target chemicals. (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research is supported by National Natural Science Foundation of China (41330637, 21422701), the Special Scientific Research Funds for Environmental Protection Commonweal Section (201309027), and National Basic Research Program of China (2015CB458900).

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