

# Differences between Lipids Extracted from Five Species Are Not Sufficient To Explain Biomagnification of Nonpolar Organic Chemicals

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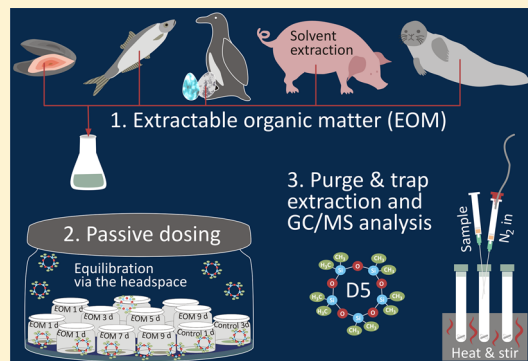
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## S Supporting Information

**ABSTRACT:** Lipids are the major sorptive phase for many organic chemicals that bioaccumulate in foodwebs. However, “lipids” are usually operationally defined by the extraction protocol. Large differences in sorptive capacities between species would violate assumptions implicit in widely used lipid-normalization procedures and invalidate generic bioaccumulation factors. We extracted lipids from five species from different trophic levels and domains and determined fractions of triglycerides, phospholipids, and cholesterol. We passively dosed the lipids with cyclic volatile methylsiloxanes and chlorobenzenes via headspace from spiked olive oil to determine their sorptive capacities. Lipids from seal blubber and pork bacon solely composed of triglycerides had capacities similar to that of olive oil; lipids from mussels, herring, and guillemot egg had quantifiable fractions of phospholipids and cholesterol and showed capacities reduced by factors of up to 2.3-fold. Generally, the sorptive capacities of the lipids were not elevated relative to the olive oil controls and are unlikely to explain a substantial part of biomagnification.



## INTRODUCTION

In bioaccumulation assessment, lipids are usually assumed to be the major reservoir for hydrophobic organic chemicals (HOCs) in organisms. What is termed “lipid”, however, is often operationally defined on the basis of the extraction procedure. The “lipid” is actually a mixture of neutral (“storage”) and polar (“membrane”) lipids, and other constituents such as sterols, which in animals consist mainly of cholesterol. Furthermore, depending on the organism’s lipid fraction and the chemical’s properties, sorptive phases other than “lipids” may be important.<sup>1,2</sup>

A recent study<sup>3</sup> reported differences in lipid-normalized whole body homogenate/water partition ratios for a range of aquatic species of up to 0.86 log unit (i.e., a factor of 7.2). Surprisingly, the extent of partitioning into the homogenates increased for organisms from higher trophic levels, which could be an indication of differences in the sorptive capacities of the lipids (and/or other relevant sorptive phases) present in the homogenates. The results of that study inspired a related Viewpoint article,<sup>4</sup> which included a call for additional research to test the hypothesis that differences in the sorptive properties of lipids might explain part of the variation observed among bioconcentration factors and even partly explain biomagnifica-

tion. While recognizing that whole body homogenates may include nonlipid sorptive phases, characterization of the affinity of HOCs for lipids, until now assumed to be uniform, was identified as being particularly important.<sup>4</sup>

The variable composition and sorptive capacities of biota lipids have been a topic of increasing attention for researchers recently. Most studies show negligible differences in the sorptive capacities of a range of pure storage lipids; for example, lipid/lipid partition ratios for HOCs were  $0.90 \pm 0.07$  ( $K_{\text{fish oil/seal oil}}$ ) and  $1.06 \pm 0.09$  ( $K_{\text{olive oil/fish oil}}$ ),<sup>5</sup> and activity coefficients for polycyclic aromatic hydrocarbons in olive oil, rapeseed oil, sunflower oil, and fish oil hardly differed.<sup>6</sup> Furthermore, the partition ratios of a large range of chemicals between five lipids (fish oil, linseed oil, goose fat, olive oil, and milk fat) and water typically varied within only  $\pm 0.1$  log unit (i.e., a factor of 1.3).<sup>7</sup> On the basis of these similarities, the chemicals’ partitioning between storage lipids and water could be described using four predictive models.<sup>8</sup>

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However, pure storage lipids are not necessarily representative of the complex lipid mixture present in biota. Therefore, the focus of this study was to extend our previous work<sup>5</sup> to passive dosing for characterization of the sorptive capacities of extractable organic matter (EOM) that consists of lipids from biota from various trophic levels. Our study hence provides a new approach to assessing the widely applied assumption that affinities of HOCs for lipids are independent of the organism.<sup>4</sup> In particular, we aimed (i) to determine how much the sorptive capacities of EOM varied among five species from different trophic levels and domains and (ii) to test the hypothesis that differences in sorptive capacities of biota lipids could explain a substantial part of the enrichment of persistent organic chemicals observed in foodwebs.

## ■ EXPERIMENTAL SECTION

**Chemicals and Solvents.** The chemicals were selected to be sufficiently volatile to allow for fast equilibration in our headspace dosing system. We investigated three cyclic volatile methylsiloxanes (cVMS) and two chlorobenzenes with octanol/air partition ratios ( $K_{OA}$ ) at room temperature in parentheses: octamethylcyclotetrasiloxane (D4, 4.3<sup>9</sup>), decamethylcyclopentasiloxane (D5, 4.9<sup>9</sup>), dodecamethylcyclohexasiloxane (D6, 5.9<sup>9</sup>), 1,2,4-trichlorobenzene (TriCB, 5.1<sup>10</sup>), and 1,2,3,4-tetrachlorobenzene (TeCB, 5.8<sup>10</sup>). Stable isotope-labeled internal standards [<sup>13</sup>C-labeled, spiked to the EOM before purge-and-trap extraction with solid-phase extraction (SPE) cartridges] were D4–D6, dichlorobenzene (used for TriCB), and pentachlorobenzene (used for TeCB). Nonlabeled polychlorinated biphenyl (PCB) 53 and Aldrin were used as recovery internal standards spiked to the SPE eluate prior to analysis.

**Samples.** To cover a wide range of trophic levels and a variety of matrices, we selected whole blue mussels (*Mytilus edulis*) without the shells, herring (*Clupea harengus*) muscle, pork (*Sus domesticus*) bacon, guillemot (*Uria aalge*) egg, and blubber of gray seal (*Halichoerus grypus*) for this study. The mussels, bacon, and olive oil were from a local store, whereas herring, guillemot egg, and seal blubber were kindly provided by the Environmental Specimen Bank (Swedish National Museum of Natural History, Stockholm, Sweden). The olive oil used to prepare the donor (see below) was used as a control of the passive dosing system and the uptake kinetics.

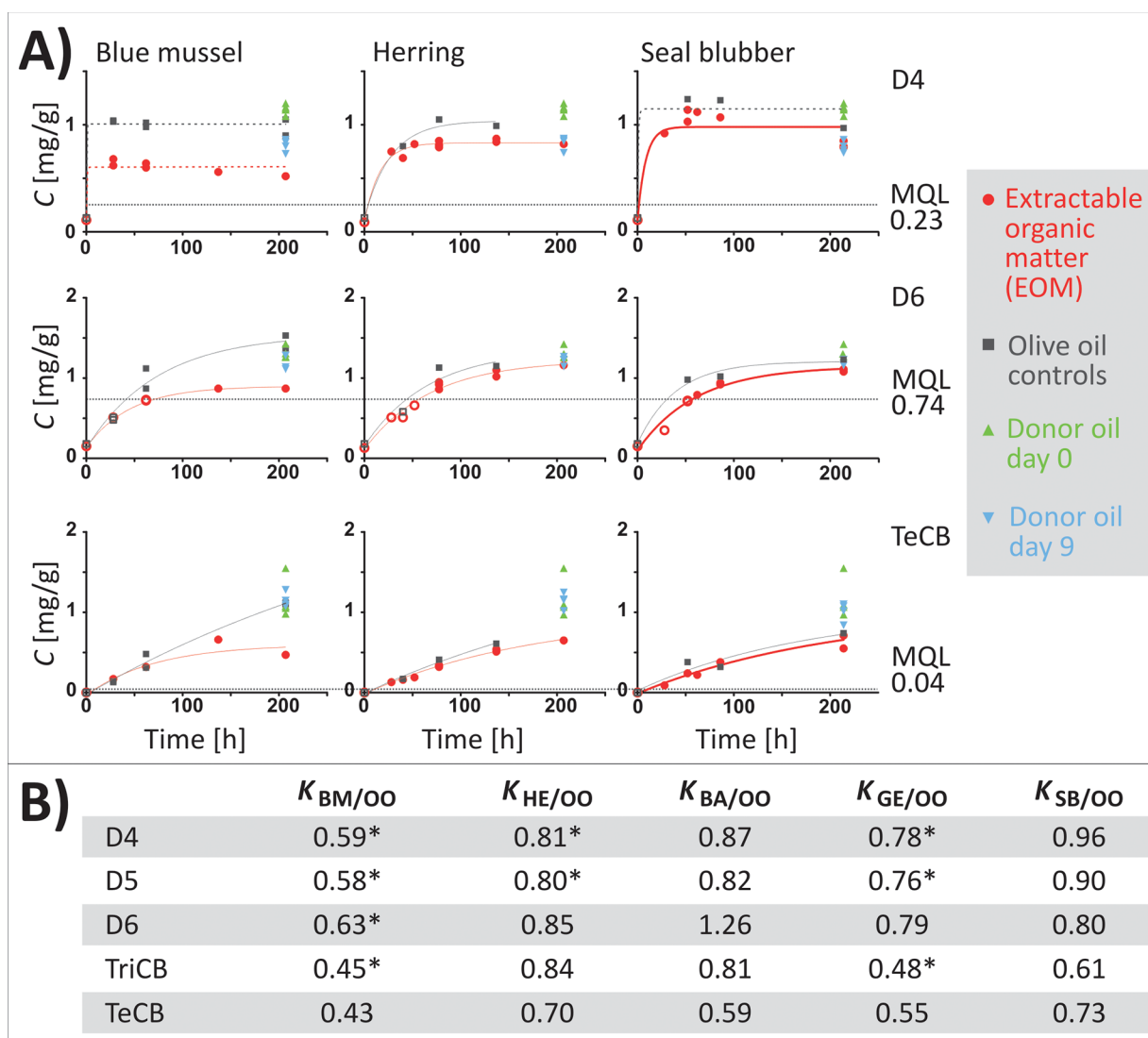
**Biota Extraction.** A traditional exhaustive solvent extraction<sup>11</sup> method was applied to extract lipids from the biotic media. Sufficient amounts of whole mussels (>40 g), herring muscle (>30 g), bacon (>3 g), and blubber (>3 g) were homogenized using a food blender and added in aliquots of up to 5 g to centrifuge tubes; 10 g of guillemot egg was processed. An *n*-hexane/acetone mixture (14 mL, 1:3) was added to each tube, and each mixture was blended, ultrasonicated (15 min), and centrifuged. The extract was transferred to a separate tube containing a NaCl/H<sub>3</sub>PO<sub>4</sub> washing solution (20 mL, 0.9%:0.1 M). The sample was reextracted with a diethyl ether/*n*-hexane mixture (14 mL, 1:9), ultrasonicated, and centrifuged, and the second extract was combined with the first in the washing solution. The tubes were rotated end over end 15 times and centrifuged. The overlying extract was quantitatively transferred to preweighed test tubes and evaporated until a constant weight was observed to allow for gravimetric determination of the EOM mass, which is frequently used as a surrogate for the lipid fraction.<sup>12</sup> The EOM was stored in the dark at 4 °C for a few days before the passive dosing experiment or the lipid

characterization was begun. For the passive dosing study, aliquots of 50 mg of EOM were added to 5 mL wide-mouth vials [ $n = 9$  per sample extract except for mussels ( $n = 6$ ) (Figure S1 of the Supporting Information)]. In addition, vials with 50 mg of olive oil were prepared (for each unit,  $n = 3$ , or  $n = 6$  in the case of mussel extract) as controls of the passive dosing kinetics and between-jar variability.

**Characterization of Extractable Organic Matter.** Two different approaches were used to characterize the EOM. A screening based on nuclear magnetic resonance (NMR) spectroscopy allowed the estimation of the samples' fractions of neutral lipids [triglycerides (TGs)], phospholipids [phosphatidylcholine (PC) and phosphatidylethanolamine (PE)], and cholesterol (CHL), giving a general picture of the composition of the extracts. More detailed studies of the EOM that did not exclusively contain neutral lipids (i.e., EOM from blue mussels, herring, and guillemot egg) were conducted after SPE fractionation<sup>13</sup> using different solvents via LC–evaporative light scattering detection (ELSD)<sup>14</sup> and GC–flame ionization detection (FID) after transmethylation.<sup>15</sup> For details, see Text S1 of the Supporting Information. Both types of lipid analyses are based on a number of assumptions and are subject to uncertainties as discussed in Text S2 of the Supporting Information. Thus, we view the two approaches used in this study as complementary.

**Passive Dosing Experiment.** Our passive dosing system (TOC Art Figure) was modified from that of ref 16. The donor oil was prepared by adding 44–56 mg of the pure chemicals to 42 g of olive oil, followed by gentle stirring for 4 days to yield a homogeneous donor phase. A 1.5 mm thick glass fiber filter was placed on the bottom of a 500 mL amber glass jar [ $n = 5$  (Figure S1 of the Supporting Information)]. Five milliliters of the donor oil was pipetted onto the filter to enlarge the donor oil's surface area and thus enhance volatilization of the model chemicals into the headspace. To each passive dosing unit were added nine vials with one type of EOM and three vials containing the unspiked olive oil controls (six and six for the mussels), and the jar opening was covered with aluminum foil and sealed with the lid. The passive dosing jars were kept at room temperature ( $21 \pm 1$  °C) and gently shaken horizontally, generating a turbulence within the jar to further enhance mass transfer of the model chemicals from the spiked olive oil into the headspace. Single, duplicate, or triplicate vials containing EOM samples were removed from the passive dosing systems after 28–214 h, allowing for the assessment of variability between replicates while ensuring the maximal time resolution for each passive dosing unit. The olive oil controls were removed over time at regular intervals.

**Purge-and-Trap Extraction and Analysis.** The extraction was conducted using a purge-and-trap method.<sup>17,18</sup> The passively dosed EOM sample was dissolved in 1 mL of dichloromethane, 10  $\mu$ L of which was transferred to a test tube (10  $\mu$ L of dichloromethane for blanks). After addition of 15  $\mu$ L of the internal standard mixture (containing all <sup>13</sup>C-labeled compounds, each at approximately 10 ng/ $\mu$ L in toluene), the tube was capped with a plug that was pierced with two cannulas (abstract graphic). An incoming stream of nitrogen, precleaned by passage through a 50 mg ENV+ cartridge, was connected to one cannula. The other cannula was coupled to a 10 mg ENV+ sampling cartridge (precleaned with 1 mL of *n*-hexane), capturing the chemicals from the outgoing stream of nitrogen. A small magnet was used to stir the EOM for the initial period (30–60 min at room temperature) until the solvent was fully



**Figure 1.** (A) Exemplary kinetics of uptake of D4, D6, and TeCB into EOM and olive oil. Broken lines represent “ambiguous” first-order kinetic uptake curves. Chemical concentrations in the donor oils from before the experiment (“day 0”) and at the end of the dosing experiment (“day 9”) are also plotted. The MQLs are indicated by dotted lines; data <MQL (empty symbols) are considered semiquantitative. The full data set is given in Figure S3 of the Supporting Information. (B) Partition ratios between EOM obtained from five different biota samples and olive oil ( $K_{EOM/OO}$ ). Cases in which the 99% confidence intervals between EOM and olive oil did not overlap are indicated by an asterisk. Abbreviations: BM, blue mussel; OO, olive oil; HE, herring; BA, bacon; GE, guillemot egg; SB, seal blubber.

evaporated. Afterward, the tube was heated to 70 °C in a heating block to push the model chemicals into the headspace and onto the ENV+ sampling cartridge. The heated extraction system was operated for at least 4 h. One blank cartridge was processed for each set of eight sample extracts. The ENV+ sampling cartridge was immediately eluted with 1 mL of *n*-hexane into an autosampler vial. The recovery standards (20  $\mu$ L of approximately 10 ng/ $\mu$ L PCB 53 and Aldrin in toluene) were added. The extracts were analyzed promptly by GC/MS (EI) (Table S1 of the Supporting Information). The method quantification limit (MQL) was defined as the average blank signal plus 10 times the standard deviation of all blanks ( $n = 11$ ). In addition, the EOM samples were analyzed at time zero, i.e., without passive dosing, to determine the concentrations of the model chemicals present in the native samples. We also conducted multiple analyses of the olive oil (nonspiked, spiked donor at time zero, and spiked donor from each passive dosing

unit after completion of the experiment) to allow for mass balance analysis.

## RESULTS AND DISCUSSION

**Characteristics of the Biota Samples.** The percentages of EOM in the five biota samples (% ww) were as follows: blue mussels, 1.4%; herring, 3.8%; pork bacon, 62%; guillemot egg, 7.0%; and seal blubber, 95%. The lipid analysis based on proton NMR spectra quantified the fractions of lipid in the EOM that were TG, PC, PE, and CHL. The NMR analysis showed that the lipids in olive oil, pork bacon, and seal blubber EOM consisted solely of TG, while EOM from blue mussel, herring, and guillemot egg contained PCs (10, 5, and 16%, respectively), PEs (10, <0.6, and 3%, respectively), and CHL (10, 1, and 5%, respectively) (Table S2 of the Supporting Information). The results obtained with the SPE-LC/ELSD and GC/FID approaches are given in Table S3 and Figure S2 of the Supporting Information. Possible changes in lipid composition



over time during the passive dosing experiment were not monitored in this study. However, the fact that the rapidly established plateau phase for D4 and D5 did not show any trends over the remaining dosing phase suggests that potential changes in lipid composition over time did not affect the EOMs' sorptive capacities. For the detailed results of the lipid characterization, see Texts S1 and S2 and Tables S2–S7 of the Supporting Information.

**Passive Dosing Study.** The MQLs were higher for the cVMS (0.23–0.74 mg/g) than for the remaining model chemicals (0.02–0.04 mg/g) because of blank contamination. Equilibration of chemicals between the olive oil donor and the EOM samples and olive oil controls in the passive dosing units was fast for the cVMS, in the range of 1–2 days for D4, 2–4 days for D5, and 4–6 days for D6 (Figure 1A and Figure S3 of the Supporting Information). TriCB and TeCB approached but did not fully reach equilibrium over the course of the passive dosing phase (9 days), and their equilibrium partitioning concentrations were hence calculated using GraphPad Prism (version 6.0) using eq 1 describing first-order kinetic uptake:

$$Y = Y_0 + (\text{plateau} - Y_0)[1 - \exp(-Kx)] \quad (1)$$

where  $x$  is the time (hours) and  $K$  is the rate constant (inverse hours). We observed chemical losses from the repeated opening of the passive dosing jars to remove vials, with the lowest overall recovery being 83% for the most volatile compound, D4, over the course of the full experiment.

**Sorptive Capacities of EOM.** Our results show differences of a factor of up to 2.3 (Figure 1B) in the sorptive capacities of the EOM obtained from biota samples spanning trophic levels and domains. Our measured partition ratios of the model chemicals between EOM and olive oil [ $K_{\text{EOM/OO}}$  (Figure 1B)] ranged from 0.43 (TeCB in mussel EOM) to 0.96 (D4 in seal blubber EOM), with one value being >1 (D6 in bacon EOM, 1.26). The sorptive capacities of EOM originating from pork bacon and seal blubber for the model chemicals were not statistically different from that of olive oil, which is consistent with all three media consisting of TGs (Table S2 of the Supporting Information). On the other hand, small but in some cases statistically significant differences were found for some chemicals in EOM obtained from blue mussels, herring, and guillemot egg compared to olive oil (Figure 1B). The largest total fractions of PCs, PEs, and CHL were found in EOM obtained from blue mussels (30% ww) and guillemot eggs (24% ww) (Table S2 of the Supporting Information), with a minor fraction also present in herring EOM (<6.6% ww), which may partly explain the observed differences. The EOM samples showed sorptive capacities slightly lower than those of the olive oil controls with no indication of elevated sorptive capacities of EOM from mussels, herring, pork bacon, or seal blubber relative to olive oil (Figure S3 of the Supporting Information). Endo et al.<sup>19</sup> found that the sorption of a large range of neutral organic compounds to PC-based artificial membrane vesicles ("liposomes") was higher than that to TGs, and that the inclusion of other membrane constituents such as CHL reduced the level of chemical partitioning into liposomes. These observations imply that one can expect minor to moderate differences in sorptive capacities of lipids between species depending on their fractions of membrane and storage lipids, and these differences seem insufficient to fully explain biomagnification.

The applied extraction method<sup>11</sup> has proven to be suitable for neutral lipids but not to be quantitative for polar lipids.<sup>20</sup>

Even though we found considerable fractions of polar lipids and sterols (Table S2 of the Supporting Information), the similarity between the sorptive capacities of the obtained EOM samples and olive oil may in part be explained by a bias as a result of the extraction method. The modified method of Jensen et al.<sup>20</sup> was specifically developed for lean biota tissue. However, a comparison of the original<sup>11</sup> and modified<sup>20</sup> "Jensen" extraction protocols that we conducted in an earlier study with a range of lean biotic media did not show substantial differences,<sup>21</sup> which is why we used the more straightforward original protocol<sup>11</sup> in this study.

The study presented here was limited to the capacities of the EOM of certain biota tissues and moderately volatile chemicals, and additional research is needed to further study when and to what extent additional constituents of organisms can contribute to the sorptive capacity for hydrophobic and bioaccumulative chemicals. To test the importance of other constituents, the dosing of whole body homogenates or intact tissues could be conducted using our passive dosing system, but such measurements are expected to be challenging because of the slower kinetics of dosing into whole tissues or their homogenates and related issues concerning sample stability. However, such measurements may become feasible provided that the dosing kinetics can be enhanced substantially, e.g., by creating more turbulence in the passive dosing system.

The maximal difference in the sorptive capacities of a broad range of lipids spanning trophic levels and domains observed in our experiments was a factor of 2.3. The differences depend on (i) the tissues that are investigated (e.g., liver tissue rich in membrane lipids vs blubber tissue rich in storage lipids vs lean muscle tissue in which proteins may be important<sup>2</sup>) and (ii) the model chemicals and may be larger for specific compounds such as H-bond donors.<sup>19</sup> The differences we observed can be a potential confounding factor when lipid-normalizing HOC concentrations in lean tissue and measured capacity differences between EOM and the reference lipid could then be used for corrections. On the other hand, the differences we observed between EOM samples are too small to account for a substantial part of biomagnification<sup>4</sup> that may encompass several orders of magnitude.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Additional material as cross-referenced throughout. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00145.

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### Notes

The authors declare no competing financial interest.

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