SPECIES-SPECIFIC DIFFERENCES IN EXTRACTABLE ORGANOCHLORINE AND ORGANOBROMINE IN HIGH-TROPHIC-LEVEL MAMMALS

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Introduction

Many unidentified organohalogen (organochlorine and organobromine) compounds are found in animals[1, 2]. The determination of organically bound chlorine (Cl) and bromine (Br) along with the individual analysis of organohalogen compounds enables us to estimate the proportions of Cl and Br mass in the identified compounds (*i.e.*, identified Cl and Br). This approach is an effective method to estimate the mass balance of identified and unidentified organohalogens and to evaluate the amounts of uniedntified organohalogen compounds. Because the cut-off value of organic molecules that can pass through cell membranes is 1,000 g/mol[3] and the molecular weight of persistent organic pollutants (POPs) is below 1,000 g/mol (decabromodiphenyl ether has the largest at 959 g/mol), low-molecular-weight compounds (< 1,000 g/mol) can include unidentified compounds with POP-like properties. POPs accumulate in high-trophic-level animals[4]. Their levels and accumulation patterns differ with species due to the species-specific metabolic capacity.[5, 6] For example, cetaceans have a lower metabolic capacity than terrestrial carnivores[6], and it has been suggested that the metabolic capacities of cats and dogs differ[7]. Therefore, we examined samples from three different high-trophic-level mammals: the striped dolphin (*Stenella coeruleoalba*), domestic cat (*Felis silvestris catus*), and raccoon dog (*Nyctereutes procyonoides*). We chose representative samples for which individual organohalogen compound ware already studied previously[8–12]. We extracted the samples using organic solvents and fractionated the extracts into low- (< 1,000 g/mol) and high-molecular-weight (≥ 1,000 g/mol) fractions. We determined the concentrations of extractable organochlorine (EOCl) and extractable organobromine (EOBr) in each fraction by neutron activation analysis (NAA). Then, the identified Cl and Br were calculated from the reported or measured concentrations of identified organohalogen compounds and the mass balance of identified/unidentified Cl and Br in the low-molecular-weight fractions were investigated.

Materials and Methods

Samples: Liver samples were collected from domestic cats ($n = 3$, all males)[8] and raccoon dogs ($n = 3$, all males)[9, 10] found dead by the roadside during 2008–2011 and 2001–2003, respectively, in Ehime, Japan. Liver samples were taken from striped dolphins ($n = 3$, all males) found stranded on Gogoshima Island, Ehime, Japan, in 2003[11, 12]. All samples were stored at −25°C in the Environmental Specimen Bank for Global Monitoring at Ehime University (Matsuyama, Japan) [13] until analysis.

Extraction, washing, and fractionation: The samples were homogenized and freeze-dried. Then, 2 g of freeze-dried sample was subsequently extracted with 10 mL of acetone, 10 mL of acetone/hexane (1:1, v/v), and 10 mL of hexane using a focused ultrasonic processor (VCX 130; Sonic & Materials, Inc.). Three fractions of each sample were combined and reduced to 10 mL by rotary evaporation (crude extract). Then, a 5-mL aliquot of crude extract (the remaining 5 mL was kept for other studies) was washed to remove the inorganic Cl and Br by partitioning the crude extracts between 5% Na_2SO_4 solution and a mixture of methyl *tert*-butyl ether and hexane (1:1, v/v). The organic phase was reduced to 10 mL by rotary evaporation and subjected to fractionation by gel permeation chromatography (GPC) with a glass column (2 cm ID × 50 cm) packed with 50 g Bio-Beads S-X3 (Bio-Rad Laboratories). The GPC column was then eluted with a mixture of cyclohexane/ethyl acetate (1:1, v/v). The first 120 mL of the eluate was considered the high-molecular-weight fraction and the second 120 mL was considered the low-molecular-weight fraction. This fractionation point was determined using a standard solution containing corn oil and a mixture of di- to deca-brominated diphenyl ethers; we considered a molecular weight of 1,000 to be the reference value for our fractionation. Each fraction was concentrated to 10 mL. Lipid contents in the samples were gravimetrically determined.

Analysis of Cl and Br: The Cl and Br concentrations were determined by NAA. First, 2 mL of fractionated extract was placed in a polyethylene (PE) bag with a paper filter and dried at normal temperature and pressure. Then, the PE bag was sealed and placed in another PE bag, which was also sealed. This was placed in a capsule and irradiated for 10 or 15 min at a thermal neutron flux of 2.0–2.4 × 10^{13} $cm^{-2}\cdot s^{-1}$ at the Kyoto University Reactor (KUR). The radioactivity of ^{38}Cl ($t_{1/2} = 37.18$ min, $E_\gamma = 1,642$ keV) and ^{80}Br ($t_{1/2} = 17.6$ min, $E_\gamma = 616$

keV) was measured for 5 min using a Ge semiconductor detector. Known concentrations of NH_4Cl and NH_4Br solution were used as standards. Quantification was based on γ-peak areas from ^{38}Cl and ^{80}Br.

Analysis of organochlorine pesticides: We analyzed organochlorine pesticides in the cat liver samples. The crude extracts were passed through a solid-phase InertSep CH cartridge (InertSep GC/PSA) for cleanup. Then, dichlorodiphenyltrichloroethane (DDT), chlordane-related compounds (CHLs), hexachlorocyclohexane isomers (HCHs), and hexachlorobenzene (HCB) were measured by gas chromatography–tandem mass spectroscopy (Agilent 7000B).

Calculation of identified Cl and Br: Cl and Br in identified compounds (identified Cl and Br) were calculated from the reported[8–12] or measured concentrations of polychlorinated biphenyls (PCBs), chlorinated pesticides (CHLs, DDT, HCHs, and HCB), and polybrominated diphenyl ethers (PBDEs).

Table 1 Concentrations (µg/g lipid) of extractable organohalogens (EOX; X = Cl or Br) in the high- (EOX-H) and low-molecular-weight (EOX-L) fractions in liver samples from three different species.

	Striped dolphin *n*=3 (male)	Domestic cat *n*=3 (male)	Raccoon dog *n*=3 (male)
Concentrations of Cl; µg/g lipid (µg/g wet in parentheses)			
EOCl[a]	240 ± 10 (7.7 ± 1.1)	210 ± 54 (8.1 ± 1.6)	170 ± 16 (5.5 ± 0.52)
EOCl-H	130 ± 12 (4.1 ± 0.90)	130 ± 42 (5.0 ± 1.4)	98 ± 8.9 (3.1 ± 0.34)
EOCl-L	110 ± 13 (3.6 ± 0.22)	80 ± 16 (3.1 ± 0.29)	77 ± 7.5 (2.4 ± 0.18)
EOCl-L/EOCl-H	0.89 ± 0.18	0.63 ± 0.13	0.78 ± 0.033
Concentrations of Cl; µg/g lipid			
Identified Cl[b]	89 ± 18	1.0 ± 0.85	12 ± 15
PCBs	23 ± 2.9	0.55 ± 0.56	0.13 ± 0.091
CHLs	12 ± 3.2	0.13 ± 0.086	12 ± 15
DDTs	51 ± 12	0.25 ± 0.16	0.037 ± 0.016
HCHs	2.4 ± 0.82	0.0085 ± 0.015	0.021 ± 0.019
HCB	1.6 ± 0.33	0.087 ± 0.051	0.0023 ± 0.0015
Identified Cl/EOCl-L (%)	79 ± 9.4	1.5 ± 1.5	16 ± 21

	Striped dolphin *n*=3 (male)	Domestic cat *n*=3 (male)	Raccoon dog *n*=3 (male)
Concentrations of Br; µg/g lipid (µg/g wet in parentheses)			
EOBr[c]	110 ± 7.8 (3.7 ± 0.71)	28 ± 2.4 (1.1 ± 0.043)	8.3 ± 3.6 (0.26 ± 0.12)
EOBr-H	51 ± 14 (1.7 ± 0.63)	25 ± 1.9 (0.98 ± 0.062)	6.6 ± 3.8 (0.21 ± 0.13)
EOBr-L	64 ± 6.6 (2.0 ± 0.14)	2.3 ± 0.80 (0.089 ± 0.022)	1.6 ± 0.32 (0.051 ± 0.0098)
EOBr-L/EOBr-H	1.3 ± 0.56	0.092 ± 0.029	0.34 ± 0.25
Concentrations of Br; µg/g lipid			
Identified Br[d]	0.57 ± 0.067	0.82 ± 0.79	0.017 ± 0.015
PBDEs	0.57 ± 0.067	0.82 ± 0.79	0.017 ± 0.015
Identified Br/EOBr-L (%)	0.90 ± 0.021	36 ± 42	1.2 ± 1.2

[a] sum of EOCl-H and EOCl-L.
[b] sum of Cl in five groups of organochlorine compounds.
[c] sum of EOBr-H and EOBr-L [d] Br in PBDEs.

Results and Discussion

Comparison with reports: We defined EOX (X = Cl or Br) in the high-molecular-weight fraction as EOX-H and EOX in the low-molecular-weight fraction as EOX-L. Table 1 shows the concentrations (µg/g wet, µg/g lipid) of EOCl-L, EOCl-H, EOBr-L, and EOBr-H. The concentrations of EOCl (EOCl-H + EOCl-L) in striped dolphin livers (240 ± 10 µg/g lipid) were similar to those reported for beluga whale (*Delphinapterus leucas*) livers (207–355 µg/g lipid)[14], while the concentrations of EOBr (EOBr-H + EOBr-L) that we determined (110 ± 7.8 µg/g lipid) were 2–5 times greater than those reported (23–48 µg/g lipid) for beluga whale livers[14]. EOBr concentrations similar to our results (3.7 ± 0.71 µg/g wet for striped dolphin and 1.1 ± 0.043 µg/g wet for domestic cat) were reported for the livers of tuna (1.1 ± 0.54 µg/g wet), albatross (1.7 ± 0.95 µg/g wet), and polar bear (2.8 ± 2.3 µg/g wet)[2].

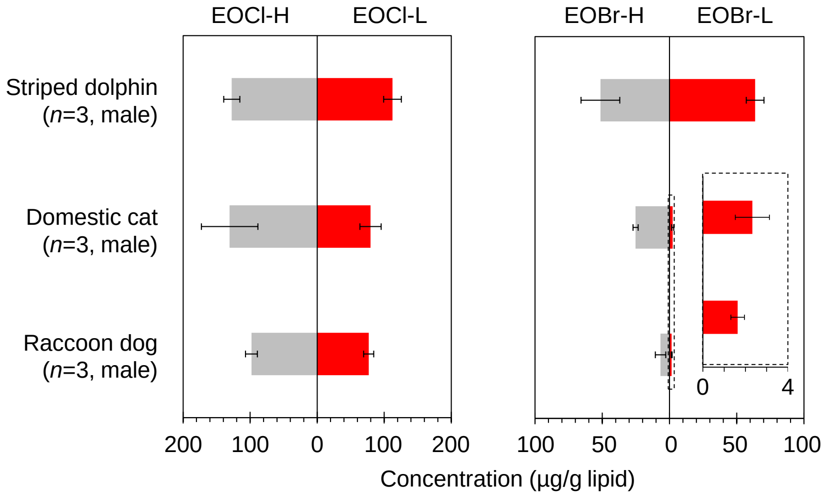

Figure 1 Concentrations (µg/g lipid) of extractable organohalogens (EOX; X = Cl or Br) in the high- (EOX-H) and low-molecular-weight (EOX-L) fractions in liver samples from three different species.

Species specific concentrations: Figure 1 shows the lipid weight-based concentrations of EOX-L and EOX-H (bars indicate the average and error bars indicate the standard deviations of the concentrations in three individual males). The EOCl concentrations exceeded the EOBr concentrations in all fractions for all species (Table 1, Figure 1). The EOCl-L concentrations did not differ among the species, ranging from 77 ± 7.5 µg/g lipid (raccoon dog) to 110 ± 13 µg/g lipid (striped dolphin), while the Cl concentrations in identified compounds (PCBs and chlorinated pesticides) differed by more than seven times (Table 1, Figure 1). This result indicates that the dominant chemical species of organochlorine compounds in these animal livers do not biomagnify like PCBs and chlorinated pesticides. The EOBr-L and EOBr-H concentrations differed markedly among the species (Figure 1). Striped dolphins had significantly higher EOBr-L levels (64 ± 6.6 µg/g), followed by the domestic cats (2.3 ± 6.6 µg/g) and raccoon dogs (1.6 ± 0.32 µg/g). EOBr-H levels were highest in the striped dolphins (51 ± 14 µg/g) followed by the domestic cats (25 ± 1.9 µg/g) and raccoon dogs (6.6 ± 3.8 µg/g). This clearly suggests that some organobromine compounds specially accumulate in the striped dolphin, including natural marine products, such as bromophenol, brominated anisole, brominated indole, and brominated dimethyl bipyrroles[15]. Greater concentrations of EOBr have also been reported in the striped dolphins previously. Kawano *et al*. reported that EOBr concentrations were 7.8–27 times greater in striped dolphins than in Dall's porpoises (*Phocoenoides dalli*) or the harbor porpoises (*Phocoena phocoena*)[16]. These observations suggest that greater EOBr concentrations are not common to all marine mammals but may be specific to the striped dolphins.

Ratio of EOX-L to EOX-H: The ratios of EOCl-L to EOCl-H (EOCl-L/EOCl-H) was similar in the three species (0.63–0.89), while the ratios of EOBr-L to EOBr-H (EOBr-L/EOBr-H) differed markedly, ranging from 0.092 to 1.3 (Table 1). Because the EOX-H concentrations were comparable or higher, studies should examine high-molecular-weight compounds, such as halogenated fatty acids (HFAs). HFAs normally contain chains with more than 16 carbon atoms[17]. HFAs are reported to contribute more than 10% to EOX[18, 19]. While HFAs produce adverse effects, fish fail to recognize HFAs as xenobiotics and incorporate them into phospholipids and triacylglycerols to approximately the same extent as stearic and oleic acids[19]. HFAs are not resistant to concentrated sulfuric acid, so theories on the bioaccumulation of POPs are not applicable to HFA[19].

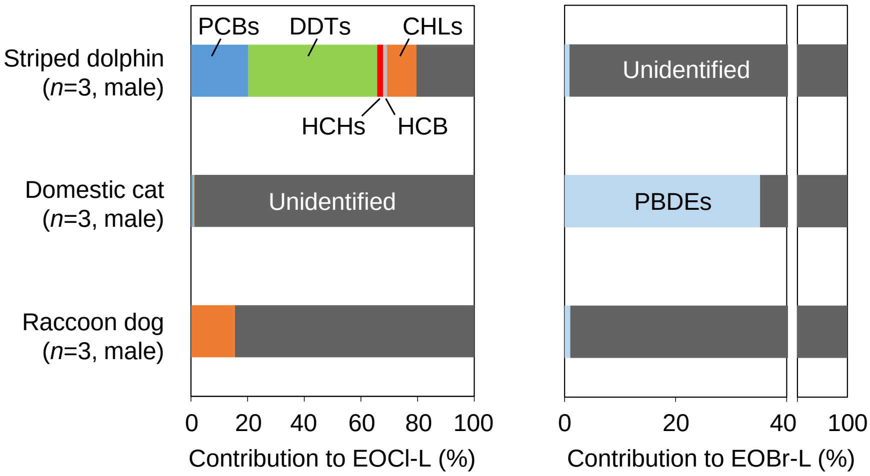

Figure 2 Contributions (%) of X in identified compounds (identified X) to EOX-L in liver samples from three different species (X = Cl or Br).

Contribution of identified compounds: Table 1 and Figure 2 show the contributions of X associated with each identified compound to EOX-L. The mean proportion of Cl contributed to EOCl-L by identified compounds (PCBs and chlorinated pesticides) was greatest in the striped dolphins (79 ± 9.4%) followed by the raccoon dogs (16 ± 21%) and domestic cats (1.5 ± 1.5%). The great contribution of identified organochlorine compounds to EOCl might be specific to cetaceans. Identified compounds (PCBs, DDT, HCHs, and CHLs) contributed 30–60% of the EOCl in the blubber of cetaceans, including the striped dolphin[12], while identified compounds (DDT and CHLs) contributed 0.2% to EOCl in the Weddell seal (*Leptonychotes weddellii*) and 2.7% to EOCl in the Adélie penguin (*Pygoscelis adeliae*) [16]. In other marine organisms, identified compounds (PCBs, HCB, HCHs, CHLs, DDT, polychlorinated naphthalenes, and polychlorinated dibenzo-*p*-dioxins and dibenzofurans) were reported to contribute 1 to 35% to EOCl: fish, 5–25%; blue crab, 35%; birds, 1–14%; and terrapin, 4.2%[1]. In the domestic cats and raccoon dogs, the concentrations of unidentified Cl were comparable to those of identified Cl in the striped dolphins. This suggests the existence of unidentified organochlorine compounds specific to the domestic cat and raccoon dog. Both external intake and biotransformation are possible sources of unidentified organochlorine compounds.

The relative proportions of identified compounds to identified Cl differed with the species. Cl associated with DDT, PCBs, and CHLs was predominant in the striped dolphins, domestic cats, and raccoon dogs, respectively. This result suggests that compounds specific to each species contribute largely to EOCl.

The proportion of Br that PBDEs contributed to EOBr-L was greatest in the domestic cats (36 ± 42%) followed by the raccoon dogs (1.2 ± 1.2%) and striped dolphins (0.90 ± 0.021%). Therefore, PBDEs are a dominant source of EOBr in domestic cats, which are exposed to PBDEs used as flame retardants because they live close to humans and ingest contaminated house dust via their grooming behavior and dietary intake[20]. The concentrations of Br associated with PBDEs were similar in the striped dolphins (0.57 ± 0.067 µg/g lipid) and domestic cats (0.82 ± 0.79 µg/g lipid), while the EOBr-L concentration was 30 times higher in the striped dolphins than in the domestic cats. Therefore, the striped dolphin is exposed to organobromine compounds other than PBDEs at much higher levels. Both natural and anthropogenic sources can be considered. Large proportions of unidentified Br have also been reported. Wan *et al*. reported that identified Br accounted for only 0.08–0.11% of the EOBr in tuna, albatross, and polar bear[2]. Since they did not fractionate EOBr by molecular weight, we cannot determine whether the unidentified Br in tuna, albatross, and polar bear is associated with high- or low-molecular-weight compounds. Applying fractionation and EOX analysis to these species should help to elucidate the character of unidentified EOX.

Conclusion

This study determined extractable organochlorine (EOCl) and organobromine (EOBr) in three different high-trophic-level mammals: the striped dolphin, domestic cat, and raccoon dog. We fractionated the EOX extracts according to molecular weight, thereby characterizing the fractions associated with high- (EOX-H) and low-molecular-weight (EOX-H) compounds. We then investigated the mass balance of identified and unidentified X in EOX-L. This showed that EOX-H and EOX-L were characteristic to each mammal species. The results also differed between Cl and Br. Unidentified organochlorine that is associated with low-molecular-weight compounds is abundant in the domestic cat and raccoon dog, while unidentified organobromine is abundant in the striped dolphin. The application of fractionation and EOX analysis in conjunction with individual analysis should help to clarify the presence and character of unidentified EOX in various animal species.

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References

1 Kannan K, Kawano M, Kashima Y, et al. (1999) *Environ. Sci. Technol*. 33(7): 1004-1008.
2 Wan Y, Jones PD, Wiseman S, et al. (2010) *Environ. Sci. Technol*. 44(16): 6068-6073.
3 Payne JW, Gilvarg C (1968) *J. Biol. Chem.* 243(23): 6291-6299.
4 Kelly BC, Ikonomou MG, Blair JD, et al. (2007) *Science*. 317(5835): 236-239.
5 Kunisue T, Tanabe S (2009) *Chemosphere*. 74(7): 950-961.
6 Nomiyama K, Murata S, Kunisue T, et al. (2010) *Environ. Sci. Technol*. 44(10): 3732-3738.
7 Mizukawa H, Nomiyama K, Nakatsu S, et al. (2013) *Environ. Pollut*. 174: 28-37.
8 Nomiyama K, Takaguchi K, Mizukawa H, et al. (2017) *Environ. Sci. Technol*. 51(10): 5811-5819.
9 Kunisue T, Takayanagi N, Isobe T, et al. (2007) *Environ. Sci. Technol*. 42(3): 685-691.
10 ChemTHEATRE: Project ID: PRA000008: https://chem-theatre.com (accessed Apr. 4, 2019).
11 Isobe T, Ochi Y, Ramu K, et al. (2009) *Mar. Pollut. Bull*. 58(3): 396-401.
12 ChemTHEATRE: Project ID: PRA000032: https://chem-theatre.com (accessed Apr. 4, 2019).
13 Tanabe S (2006) *J. Environ. Monit*. 8(8): 782-790
14 Kiceniuk JW, Holzbecher J, Chatt A (1997) *Environ. Pollut*. 97(3): 205-211.
15 Alonso MB, Maruya KA, Dodder NG, et al. (2017) *Environ. Sci. Technol*. 51(3): 1176-1185.
16 Kawano M, Falandysz J, Tanaka Y, et al. (1997) *Organohalogen Compd*. 33: 328-332.
17 Dembitsky VM, Srebnik M (2002) *Prog. Lipid Res*. 41(4): 315-367.
18 Mu H, Wesén C, Sundin P (1997) *Trac-Trends Anal. Chem*. 16(5): 266-274.
19 Mu H, Ewald G, Nilsson E, et al. (2004) *Environ. Sci. Technol*. 38(21): 5548-5554.
20 Guo W, Park JS, Wang Y, et al. (2012) *Environ. Toxicol. Chem*. 31(2): 301-306.