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Identification of Major Dioxin-Like Compounds and Androgen Receptor Antagonist in Acid-Treated Tissue Extracts of High Trophic-Level Animals

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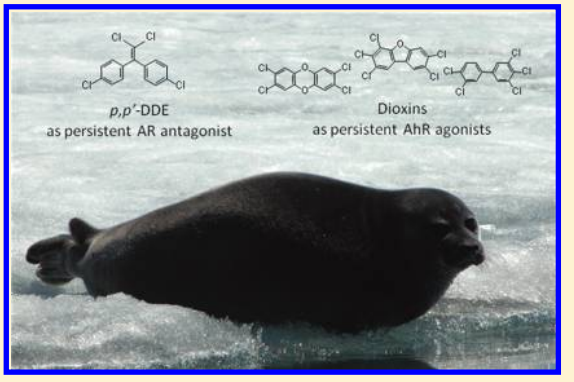
Supporting Information

ABSTRACT: We evaluated the applicability of combining *in vitro* bioassays with instrument analyses to identify potential endocrine disrupting pollutants in sulfuric acid-treated extracts of liver and/or blubber of high trophic-level animals. Dioxin-like and androgen receptor (AR) antagonistic activities were observed in Baikal seals, common cormorants, raccoon dogs, and finless porpoises by using a panel of rat and human cell-based chemical-activated luciferase gene expression (CALUX) reporter gene bioassays. On the other hand, no activity was detected in estrogen receptor α (ERα)-, glucocorticoid receptor (GR)-, progesterone receptor (PR)-, and peroxisome proliferator-activated receptor γ2 (PPARγ2)-CALUX assays with the sample amount applied. All individual samples (n = 66) showed dioxin-like activity, with values ranging from 21 to 5500 pg CALUX-2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent (TEQ)/g-lipid. Because dioxins are expected to be strong contributors to CALUX-TEQs, the median theoretical contribution of dioxins calculated from the result of chemical analysis to the experimental CALUX-TEQs was estimated to explain up to 130% for all the tested samples (n = 54). Baikal seal extracts (n = 31), but not other extracts, induced AR antagonistic activities that were 8−150 μg CALUX-flutamide equivalent (FluEQ)/g-lipid. *p,p′*-DDE was identified as an important causative compound for the activity, and its median theoretical contribution to the experimental CALUX-FluEQs was 59% for the tested Baikal seal tissues (n = 25). Our results demonstrate that combining *in vitro* CALUX assays with instrument analysis is useful for identifying persistent organic pollutant-like compounds in the tissue of wild animals on the basis of *in vitro* endocrine disruption toxicity.



INTRODUCTION

Environmental pollution by anthropogenic chemicals is one of the most pressing global problems. More than 60 million industrial chemicals have been produced up to now.[1] It is estimated that large numbers of these artificial compounds have been released to the environment and may have been accumulated in the tissues of wild animals, such as top predators, via the food web. The toxic effects of these compounds, such as endocrine disruption, need to be identified and monitored, particularly in the case of top predators, which include both wildlife and humans. However, our lack of information on the toxicity of many of these compounds limits our ability to selectively monitor the most toxic ones. Furthermore, recent studies have indicated the existence of hidden potential toxicants, such as persistent metabolites,[2−4] impurities in chemical products,[5−8] and unintentional byproducts from physicochemical changes such as thermal/photolytic decomposition.[9,10] Consequently, from a toxicological point of view there is a need for effective chemical assessment schemes such as toxicity identification evaluation (reviewed by Burgess[11]) and effect-directed analysis (reviewed by Brack et al.[12]).

Our research has been focused on developing a strategic methodology by using *in vitro* bioassay together with qualitative/quantitative chemical analysis to determine *in vitro* toxicity

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Table 1. General Information of the Tested Wild Animal Tissues

species	scientific name	tissue	lipid (%)	*n*	age (year)	sampling year	sampling location
Baikal seal	*Phoca sibirica*	blubber	94 (89−96)	10 male	17 (0.25−42)	2005	Lake Baikal, Russia
		liver	3.8 (2.8−4.4)				Lake Baikal, Russia
		blubber	87 (72−98)	6 male	13 (1.5−25.5)	1992	Lake Baikal, Russia
		liver	5.2 (36−5.9)				Lake Baikal, Russia
common cormorant	*Phalacrocorax carbo*	liver	4.9 (4.2−5.9)	5 female	not available	2002	Lake Biwa, Japan
			5.2 (4.4−6.1)	5 male	not available	2002	Lake Biwa, Japan
raccoon dog	*Nyctereutes procyonoides*	liver	4.2 (3.5−5.5)	4 female	not available	2001	Kanagwa, Japan
			4.0 (3.2−4.6)	6 female	not available	2001	Kanagwa, Japan
finless porpoise	*Neophocaena phocaenoides*	liver	4.6, 8.1	2 unknown	not available	2005	Oita, Japan
			4.6, 24	2 unknown	not available	2005	Nagasaki, Japan
			5.8	1 male	not available	2005	Nagasaki, Japan
			9.6	1 male	not available	2006	Nagasaki, Japan
			4.0	1 male	not available	2007	Ehime, Japan
			4.2	1 female	not available	2007	Oita, Japan
			6.9	1 male	not available	2007	Hyogo, Japan
			21	1 female	not available	2007	Hyogo, Japan

profiles and potential bioaccumulative pollutants in wild animal populations. The technique applied in this study, which uses cell-based chemical-activated luciferase gene expression (CALUX) reporter gene bioassays for high trophic-level animals, has at least two advantages as an effective assessment scheme: (1) it targets high trophic-level animals in their ecosystems and thus covers bioaccumulation through the ecological chain as well as through generated xenometabolic reactions, targeting a wide variety of potential contaminants; and (2) CALUX reporter gene bioassays, especially with androgen receptor (AR), estrogen receptor α (ERα), progesterone receptor (PR), and dioxin receptor (DR) CALUX,[13−16] clearly indicate that there are suitable and reliable detection tools for AR, ERα, PR, and DR agonists/antagonists.

In this study, we used a panel of rat and human cell-based CALUX reporter gene bioassays to evaluate steroid hormone-disrupting potency (AR, ERα, glucocorticoid receptor [GR], and PR-mediated activities), dioxin-related toxicity (aryl hydrocarbon receptor [AhR]-mediated activity), and lipid metabolism-disrupting potency (peroxisome proliferator-activated receptor γ2 [PPARγ2] -mediated activity) in blubber and/or liver extracts from four top predators (Baikal seal [*Puca sibirica*], common cormorant [*Phalacrocorax carbo*], raccoon dog [*Nyctereutes procyonoides*], and finless porpoise [*Neophocaena phocaenoides*]) to identify important compounds indicating *in vitro* endocrine disruption toxicity. Here, we describe the detection of persistent organic pollutant (POP)-like compounds in these predators.

EXPERIMENTAL SECTION

Animal Samples. In this study, we evaluated not only blubbers but also livers from wild animals using a strategic methodology combining *in vitro* bioassay together with qualitative/quantitative chemical analysis. Although POP-like compounds tend to accumulate in blubber and liver, their metabolites are likely to be produced in livers but not blubber. Therefore, this study was designed with livers as the principal target tissue.

We obtained permission issued by the Lake Baikal Basin Committee for Protection, Reproduction of Fish Resources and Fishing Control (known by its Russian acronym BAIKALRYBVOD) under the annual seal culling quota to collect Baikal seals from Lake Baikal in Russia in 1992[17,18] and 2005,[17,18] and their blubber (1992, n = 10; 2005, n = 10) and livers (1992, n = 6; 2005, n = 10) were analyzed. Three other animals were sampled from Japan: common cormorants from Lake Biwa in 2002,[19] which had been removed from the lake for being destructive birds; raccoon dogs killed by traffic in Kanagawa in 2001,[20] and finless porpoises stranded in Hyogo, Ehime, Oita, and Nagasaki between 2005 and 2007.[21] Their livers (each n = 10) were extracted for analyses. All sampled tissues were stored at −25 °C in the Environmental Specimen Bank (*es*-BANK) at Ehime University[22] until analysis. General sample information is listed in Table 1.

Extraction and Cleanup. Ten grams of blubber or liver sample was extracted with an acetone/*n*-hexane mixture (1:1 v/v) using a rapid solvent extractor (SE-100; Mitsubishi Chemical Analytech, Chigasaki, Japan). The extract was solvent exchanged into *n*-hexane as a crude extract. One-fifth of the crude extract for liver samples or 0.05 for blubber samples was treated with concentrated sulfuric acid. The n-hexane fraction was washed with water, dehydrated, and then applied to a cleanup column composed of sodium sulfate, 22% (w/w) sulfuric acid silica gel, 44% (w/w) sulfuric acid silica gel, and sodium sulfate (in top down order). After elution with n-hexane, the extract was evaporated, and the residue was redissolved in 100 μL of dimethylsulfoxide (DMSO). We defined this treated extract as the persistent extract containing persistent chemicals that would not be decomposed by sulfuric acid treatment. For screening purposes, a pooled extract was prepared for each species/tissue by mixing the persistent extracts of the respective tissues. Individual extracts were also prepared and evaluated in detail for the activities detected by screening. All extracts were stored at 4 °C until *in vitro* bioassay analysis.

To determine the recovery of the cleanup procedure for major target compounds, n-hexane was spiked with 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD) and 3,3′,4,4′,5′-pentachlorobiphenyl (PCB#126) as dioxin-like compounds, and 1,1-dichloro-2,2-bis (p-chlorophenyl)ethylene (*p,p*′-DDE) as AR antagonist identified in this study for subsequent the above-mentioned cleanup. Target compounds were quantified by DR-CALUX and AR-CALUX assay, respectively. Recovery of 1,2,3,7,8-PeCDD, PCB#126, and *p,p*′-DDE ranged between 79% and 110%.

Screening of Endocrine-Disrupting Chemicals Using in Vitro Bioassays. A panel of *in vitro* rat and human cell-based CALUX reporter gene bioassays was used for screening of bioaccumulated compounds in acid-treated animal tissue extracts. The characteristics of steroidal hormone-disrupting potency (AR, ERα, GR, and PR-mediated agonistic and antagonistic activities), dioxin-related toxicity (AhR-mediated agonistic and antagonistic activities), and lipid metabolism-disrupting potency (PPARγ2-mediated agonistic and antagonistic activities) were investigated to assess potential hazardous chemicals in wildlife.

AR-, ERα-, GR-, PR-, and PPARγ2-CALUX Assays. AR-, ERα-, GR-, PR-, and PPARg2 CALUX cell lines were human osteosarcoma cell lines stably cotransfected with target human receptor−regulated luciferase gene construct (U2OS-*luc* cells) developed by BioDetection Systems b.v. (Amsterdam, The Netherlands). The assay procedure with U2OS-*luc* cells basically followed the BDS method.[14−16,23] U2OS-*luc* cells were continuously cultured at 37 °C under 7.5% CO_2 and high humidity in DF (1:1 Mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 [DMEM/F12]) medium supplemented with 7.5% fetal calf serum (FCS). U2OS-*luc* cells were seeded into a 96-well microplate with DF medium (without phenol red) supplemented with stripped (dextran-coated and charcoal-treated) FCS. After 24 h of incubation, the medium was replaced by medium with stripped FCS containing reference chemicals and/or sample extracts for agonistic and antagonistic response testing, as described below. After 24 h of exposure, the medium was removed, and the cells were lysed in Triton-lysis buffer. Luciferin solution was then added, and the luciferase activity was measured with a luminometer (Lucy 2, Anthos, Austria).

DR-CALUX Assay. The rat hepatoma H4IIE cell line (DR-CALUX cells), stably transfected with a rat AhR-regulated luciferase gene construct, was also obtained from BDS. The conditions for cell culture and the assay procedure have been described in detail elsewhere.[24] In short, DR-CALUX cells were subcultured at 37 °C under 5% CO_2 and high humidity in α-minimal essential medium (αMEM) supplemented with 10% FCS. DR-CALUX cells were seeded in a 96-well microplate with αMEM supplemented with 10% FCS, and cells with 90% to 95% confluence were used for exposure. After 24 h, cells were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; AccuStandard, New Haven, CT, USA) or to sample extracts for agonistic testing, as described below. After 24 h of exposure, the medium was removed, and the cells were washed twice with phosphate-buffered saline without calcium and magnesium ions. Each well was filled with Triton-lysis buffer. After addition of luciferin solution, the luciferase activity was measured with a luminometer. Antagonistic testing with DR-CALUX was not performed in this study because of the high agonistic activity of all extracts.

Agonistic Testing. The cells were exposed to reference agonists, such as 2,3,7,8-TCDD for DR-CALUX, or to sample extracts. Levels of luciferase induction by the sample extracts were expressed in terms of the percentage of the maximum level of induction by the respective reference agonist. In this study, extracts with more than 10% induction were judged as agonistic.

Antagonistic Testing. Three types of experiment were performed as antagonistic testing. In the first, the cells were exposed to the sample extract or reference antagonist (e.g., flutamide [Sigma-Aldrich, San Diego, CA, USA] for AR-CALUX cells) as well as a reference agonist (e.g., dihydrotestosterone [DHT; WAKO, Osaka, Japan] for AR-CALUX cells) at the median effective concentration (EC_{50}) level in the same exposure medium (antagonist$_{EC50}$ assay). In this experiment, luciferase induction levels of cells exposed to sample extracts were expressed in terms of the percentage of the level in cells exposed to DMSO vehicle control at the EC_{50} level of the reference agonist. If the induction level was less than 80%, cell cytotoxicity in the same exposure medium was investigated in the second type of experiment with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the procedure described in detail elsewhere.[23] An inhibitory effect was judged as antagonistic in case of no cytotoxic effect. In the third type of experiment, the cells were exposed to not only the sample extract or reference antagonist but also the 100 × EC_{50} level of reference agonist in the same exposure medium (antagonist$_{EC50\times100}$ assay). Antagonistic effects were considered as receptor specific if mitigated by coexposure to a high dose of reference agonist.

Quantitative Analysis with in Vitro Bioassay. Agonistic Activity. An agonistic response on DR-CALUX cells, but not other cell lines, was observed in this study. The calibration dose−response curve for 2,3,7,8-TCDD standards (blank, 0.3, 1.0, 3, 10, 30, 100, 300 pM/well in DMSO) was fitted to a three-parameter sigmoid by using Slide Write Plus software version 6.00 (Advanced Graphics Software, Encinitas, CA, USA): $y = a_0/[1 + (x/a_1)^{a2}]$, where y = measured luciferase induction; x = concentration; a_0 = maximum luciferase induction (approximately 100%); $a_1 = EC_{50}$; and a_2 = curve slope. The limit of detection and the limit of quantification were less than 0.3 and 1 pM, respectively. The CALUX-TCDD equivalent (CALUX-TEQ) per gram lipid of sample was calculated from the activities between those of 1 and 4 pM 2,3,7,8-TCDD (after correction for background activity of the DMSO control) by interpolation on the fitted 2,3,7,8-TCDD calibration curve. Each measurement was performed in three wells on the same microplate and repeated at least once.

Antagonistic Activity. Antagonistic response was observed on AR-CALUX cells but not on the other cells. The CALUX-flutamide equivalents (CALUX-FluEQs) per gram lipid of sample were calculated from luciferase induction levels of less than 80% (20% inhibition or more) by interpolation of the fitted dose−response curves for the flutamide standard. A calibration dose−response curve for flutamide standards (blank, 1, 3, 10, 30, 100, 300, 1000, 3000 nM/well in DMSO) was fitted to a four-parameter sigmoid by using SigmaPlot for Windows Version 11.0, Systat Software, Inc., San Jose, CA, USA): $y = min + (max - min)/(1 + 10^{(\log\ a1-x)} \times a_2)$, where y = measured luciferase induction; x = concentration; min = luciferase induction with the maximum concentration of flutamide standard (approximately 0%); max = luciferase induction with DMSO vehicle control (approximately 100%); $a_1 = IC_{50}$ (half-maximum inhibitory concentration); and a_2 = curve slope. Each measurement was conducted in three wells on the same microplate and repeated at least once independently.

Identification of Causative AR Antagonists by GC-MSD. An aliquot of the acid-treated extract with potent AR antagonistic activity was extracted with *n*-hexane after addition of water. The *n*-hexane fraction was dehydrated using sodium sulfate and concentrated to a small volume for identification by using GC-MSD (Agilent Technologies 6890N series GC, equipped with a 5973N series Mass Selective Detector; Agilent, Wilmington, DE, USA) and a SGE-BPX5 column (25 m × 0.22 mm i.d., 0.3-μm film thickness; SGE, Darmstadt, Germany).[42] For tentative identification, the mass spectra obtained were compared with reference spectra in the US National Institute of Standards and

Criteria	Luciferase induction	<5	<10	<30	<70	<100		
2,3,7,8-TCDD	pM/well	3.E-01	1.E+00	3.E+00	1.E+01	3.E+01	1.E+02	3.E+02
	Luciferase induction / Agonist assay	<5	7.1	25	60	85	97	100
Baikal seal (2005), blubber	g-lipid/well	4.E-06	1.E-05	4.E-05	1.E-04			
	Luciferase induction / Agonist assay	<5	<5	9.1	21			
Baikal seal (1992), blubber	g-lipid/well	4.E-06	1.E-05	4.E-05	1.E-04			
	Luciferase induction / Agonist assay	<5	6.8	17	34			
Baikal seal (2005), liver	g-lipid/well	7.E-07	2.E-06	7.E-06	2.E-05			
	Luciferase induction / Agonist assay	<5	<5	12	26			
Baikal seal (1992), liver	g-lipid/well	1.E-06	3.E-06	1.E-05	3.E-05			
	Luciferase induction / Agonist assay	5.2	12	24	49			
Common cormorant, liver	g-lipid/well	8.E-07	3.E-06	8.E-06	3.E-05			
	Luciferase induction / Agonist assay	<5	12	25	48			
Raccoon dog, liver	g-lipid/well	7.E-07	2.E-06	7.E-06	2.E-05			
	Luciferase induction / Agonist assay	<5	7.0	15	33			
Finless porpoise, liver	g-lipid/well	2.E-06	6.E-06	2.E-05	6.E-05			
	Luciferase induction / Agonist assay	<5	<5	6.1	12			

Figure 1. Agonistic dose responses for induction of luciferase activity in the DR-CALUX assay using 2,3,7,8-TCDD and pooled sulfuric acid-treated extracts of wild animal tissues. Luciferase induction is expressed in terms of the percentage of the luciferase activity of 300 pM 2,3,7,8-TCDD. Color strength indicates DR agonistic potency according to the criteria. Values represent percentage inductions of at least two independent assays.

Technology (NIST) main mass spectral database, and a match value greater than 80% was considered as indicating a good match.

RESULTS AND DISCUSSION

***In Vitro* Endocrine-Disrupting Activities in Tissues of Wild Animals.** A screening of seven pooled persistent extracts prepared from blubber and liver tissues from the four sampled species showed marked dose-dependent DR agonistic activity (more than 10% induction) in all samples (Figure 1). Marked AR antagonistic activity (more than 20% inhibition) was detected only in extracts prepared from Baikal seal tissues (Figure 2), where it was dose dependent, whereas there was no activity in ERα-, GR-, PR-, and PPARγ2-CALUX assays with the sample amount applied. We observed no cytotoxic effects in the same exposure medium with the antagonist$_{EC50}$ assays (data not shown) nor any marked decrease in luciferase induction in the antagonist$_{EC50\times100}$ assays (Figure 2), thus confirming that the pooled persistent extracts of Baikal seal tissues were AR-specifically antagonistic. These findings suggest that accumulative compounds associated with dioxin-like toxicity and AR antagonistic effects should be evaluated in detail from an *in vitro* toxicological point of view.

Quantitative Evaluation of Activity Levels Detected in Individual Persistent Extracts. *Dioxin-Like Activity.* All individual persistent extracts of wild animal tissues had marked dioxin-like activity in the pooled extracts. Dioxin-like activities were quantified as CALUX-TEQs in each persistent extract under reproducible assay conditions, as described below. Average dose responses for luciferase induction by 2,3,7,8-TCDD in the DR-CALUX assays (n = 64) are shown in Figure S1. The calculated EC_{50} of the 2,3,7,8-TCDD standard for the DR-CALUX assay was 7.6 ± 1.6 pM in a microplate well (average ± standard deviation [SD]). The average maximum induction (i.e., luciferase activity of 300 pM 2,3,7,8-TCDD in the DR-CALUX cells divided by luciferase activity induced by DMSO) was 10.5 ± 2.2 (average ± SD). The results of average EC_{50} and maximum induction satisfied the quality levels indicated in the standard operating procedure provided by the supplier of the DR-CALUX cells (BioDetection Systems b.v.) and were consistent with those obtained in our previous study.[25] Reproducible CALUX-TEQs in the persistent extracts were 55 to 720 pg/g-lipid (median 190 pg/g-lipid) for blubber samples of Baikal seals (n = 20), 370 to 5100 pg/g-lipid (median 1700 pg/g-lipid) for liver samples of Baikal seals (n = 16), 1300 to 5500 pg/g-lipid (median 2600 pg/g-lipid) for liver samples of common cormorants (n = 10), 440 to 3500 pg/g-lipid (median 650 pg/g-lipid) for liver samples of raccoon dogs (n = 10), and 21 to 300 pg/g-lipid (median 230 pg/g-lipid) for liver samples of finless porpoises (n = 10). Because dioxins were expected to be strong contributors to CALUX-TEQs, we compared these experimental CALUX-TEQ values with TEQs derived from previously reported dioxin data[17−21] (see below).

AR Antagonistic Activity. The 16 blubber and 15 liver persistent extracts of Baikal seal indicated marked AR antagonistic activity. In contrast, extracts from four blubber samples and one liver sample had no antagonistic effect as pooled extracts with the amounts of samples applied. Bioaccumulative AR antagonists were quantified as CALUX-FluEQs in individual persistent extracts from Baikal seals under reproducible assay conditions, as described below. The average dose responses for the inhibition of

Criteria	Luciferase induction	0<	50<	60<	70<	80<

Flutamide	nM/well		1.E+00	3.E+00	1.E+01	3.E+01	1.E+02	3.E+02	1.E+03	3.E+03
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	80<	80<	80<	77	51	20	5
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<	80<	80<	80<	80<	80<

Baikal seal (2005), blubber	g-lipid/well		8.E-05	3.E-04	8.E-04
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	64	49
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<
Baikal seal (1992), blubber	g-lipid/well		6.E-05	2.E-04	6.E-04
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	62	56
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<
Baikal seal (2005), liver	g-lipid/well		5.E-07	2.E-06	5.E-06
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	80<	78
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<
Baikal seal (1992), liver	g-lipid/well		1.E-06	3.E-06	1.E-05
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	76	73
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<
Common cormorant, liver	g-lipid/well		8.E-07	8.E-06	8.E-05
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	80<	80<
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<
Raccoon dog, liver	g-lipid/well		8.E-07	8.E-06	8.E-05
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	80<	80<
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<
Finless porpoise, liver	g-lipid/well		2.E-05	6.E-05	2.E-04
	Luciferase induction	Antagonist$_{EC50}$ assay	80<	80<	80<
		Antagonist$_{EC50\times100}$ assay	80<	80<	80<

Figure 2. Antagonistic dose responses for inhibition of luciferase activity in the AR-CALUX assay using flutamide and pooled sulfuric acid-treated extracts of wild animal tissues at EC_{50} and $EC_{50} \times 100$ of DHT. Luciferase induction is expressed in terms of the percentage of the luciferase activity induced by a DMSO vehicle control. Color strength indicates AR antagonistic potency according to the criteria. Values represent percentage inductions of at least two independent assays.

luciferase induction by flutamide in the AR-CALUX assays ($n = 10$) at EC_{50} and $100 \times EC_{50}$ levels of DHT as the reference agonist are shown in Figure S2. The calculated IC_{50} of the flutamide standard at the EC_{50} level of DHT was 270 ± 48 nM in a microplate well (average $\pm$ SD), comparable to the values reported in the validation study for this reporter gene assay by BioDetection Systems b.v.[14] As reproducible results, the CALUX-FluEQs of Baikal seal persistent extracts ranged from not detected (ND) to 110 μg/g-lipid (median 21 μg/g-lipid) for blubber samples ($n = 20$) and ND to 150 μg/g-lipid (median 62 μg/g-lipid) for liver samples ($n = 16$). Four of 20 blubber samples indicated ND levels that ranged between 5 to 7 μg CALUX-FluEQ/g-lipid. These lower values were derived from younger Baikal seals with ages ranging from 0.25 to 4.5 years. Persistent extracts with AR antagonistic activity were prepared from older Baikal seals (10.5 to 41.5 years old). AR antagonistic activity in the liver samples was not detected in one (ND: 72 μg CALUX-FluEQ/g-lipid) of 16 samples because of the small size of the sample used in the exposure experiment. There was a relatively good positive correlation between AR antagonistic potency and Baikal seal age ($r = 0.58$, $p = 0.0015$) (Figure S3), suggesting that the major contributors to these activities were accumulative compounds with POP-like behavior. However, there were many candidate causative compounds,[26−31] such as persistent pesticides, plastic additives, and engineering products (e.g., polychlorinated biphenyls; PCBs), in the tested extracts. Therefore, pooled persistent extracts of Baikal seal tissues were analyzed with GC-MSD for quantitative evaluation of the causative chemicals, as described below.

Toxic Identification and Evaluation. *Dioxin-Like Compounds.* To evaluate the contribution of dioxin-like compounds to the DR-CALUX-measured dioxin-like activities in persistent extracts of wild animal tissues, previously reported concentrations of polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDD/Fs) and coplanar-polychlorinated biphenyls (Co-PCBs) (Table S1) for blubber (1992, $n = 10$; 2005, $n = 10$)[17] and livers (2005, $n = 10$; Imaeda et al., unpublished data) of Baikal seals, and for livers of common cormorants ($n = 10$),[19] raccoon dogs ($n = 10$),[20] and finless porpoises ($n = 4$),[21] were used to calculate the theoretical CALUX-TEQs by multiplying by the respective CALUX potencies relative to TCDD (Table S2),[13] as described in our previous study.[25] The theoretical CALUX-TEQs for PCDD/Fs and Co-PCBs in the persistent extracts were 21 to 310 pg/g-lipid (median 87 pg/g-lipid) and 59 to 460 pg/g-lipid (median 140 pg/g-lipid), respectively, for blubber samples of Baikal seals ($n = 20$); 100 to 1900 pg/g-lipid (median 340 pg/g-lipid) and 170 to 760 pg/g-lipid (median 390

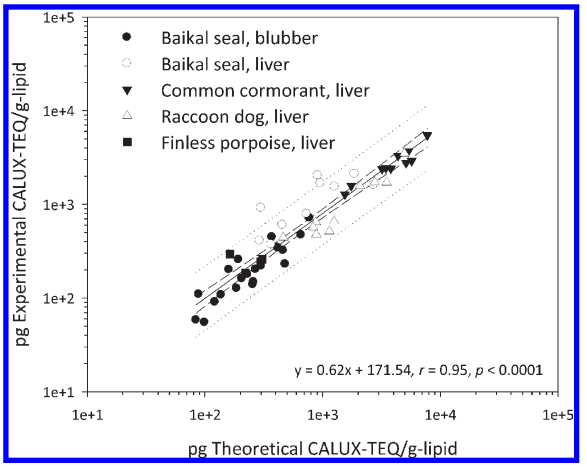

Figure 3. Experimental CALUX-TEQs and theoretical values calculated by using PCDD/F and co-PCB concentrations (see Table S1)[17,19−21] and the corresponding DR-CALUX potencies relative to that of 2,3,7,8-TCDD (see Table S2)[13] in blubber of Baikal seals (n = 20) and livers of Baikal seals (n = 10), common cormorants (n = 10), raccoon dogs (n = 10), and finless porpoises (n = 4). Correlation coefficient was calculated by using a nonparametric Spearman correlation. The solid line represents the fitted line, and the short dash lines indicate the 95% confidence interval for the fit. Dotted lines indicate the prediction interval at 95% confidence.

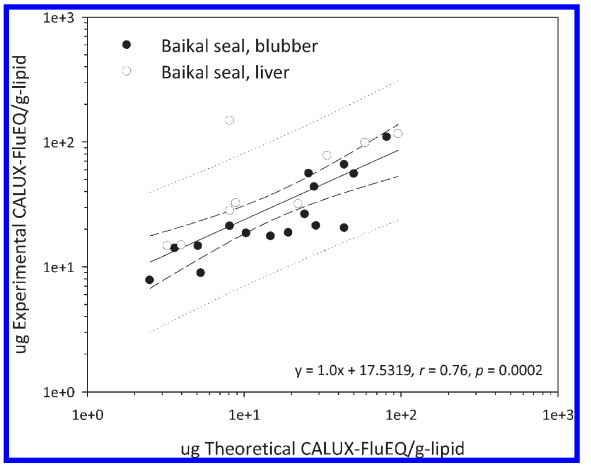

Figure 4. Experimental CALUX-FluEQs and theoretical values calculated by using p,p'-DDE concentrations (see Table S4)[37] and AR-CALUX potency relative to flutamide (see Table S3) in blubber (n = 20) and liver (n = 10) of Baikal seals. Correlation coefficient was calculated by using a nonparametric Spearman correlation. The solid line represents the fitted line, and the short dash lines indicate the 95% confidence interval for the fit. Dotted lines indicate the prediction interval at 95% confidence.

pg/g-lipid) for liver samples of Baikal seals (n = 10); 1000 to 4200 pg/g-lipid (median 2300 pg/g-lipid) and 490 to 3800 pg/g-lipid (median 1600 pg/g-lipid) for liver samples of common cormorants (n = 10); 420 to 3900 pg/g-lipid (median 1100 pg/g-lipid) and 30 to 1000 pg/g-lipid (median 130 pg/g-lipid) for liver samples of raccoon dogs (n = 10); and 150 to 290 pg/g-lipid (median 240 pg/g-lipid) and 16 to 21 pg/g-lipid (median 19 pg/g-lipid) for liver samples of finless porpoises (n = 4). The correlation between the total theoretical and the experimental CALUX-TEQs (Figure 3) indicates that internationally regulated dioxins, such as PCDD/Fs and Co-PCBs,[32] could in large part explain the whole dioxin-like activity in persistent extracts of the tested animal tissues. The median theoretical contribution of dioxins to the experimental CALUX-TEQ was 130% (blubber of Baikal seal), 78% (liver of Baikal seal), 140% (common cormorant), 150% (raccoon dog), and 120% (finless porpoise). The theoretical CALUX-TEQs tended to be higher than the respective experimental values in tested samples. This difference might have been due to the effects of partial AhR agonists[33] or antagonists[34] that had accumulated in these animal tissues. Another possible reason for the overestimation of the theoretical CALUX-TEQs might have been related to the recovery rates of the target compounds, which were taken into account in the chemical analyses using internal standards but not in the bioassays. We previously[25,35] eliminated this influence by using the same sulfuric acid-treated extracts for comparative evaluation of the experimental and theoretical CALUX-TEQs. In the present study, original samples, but not sulfuric acid-treated extracts, were used between the CALUX assays and instrument analyses, and this has been one reason for the overestimation of the theoretical CALUX-TEQs. However, the results for the livers of Baikal seals and finless porpoises suggest that unidentified dioxin-like compounds, such as dioxin-like polybrominated dibenzo-p-dioxins and dibenzofurans and polychlorinated naphthalene,[13] exist in these tissues. Despite this, our results clearly indicated that internationally regulated dioxins such as POPs were the important contributors to the experimental CALUX-TEQs for all tested animal samples.

AR Antagonists. Because there are potentially many accumulated AR antagonists in wild animals,[26−31] the pooled persistent extracts of Baikal seal tissues were analyzed by using GC-MSD with electron-impact (EI) ionization to identify the causative compounds of the AR antagonistic responses. A potent peak was detected in all tested pooled persistent extracts. We compared the EI mass spectra of this potent peak with the NIST reference spectra and tentatively identified the compound as p,p'-DDE, which is one of the major 1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane (p,p'-DDT) transformation products and one of the first pollutants categorized as a POP. From a toxicological point of view, this compound is also a well-known potent true AR antagonist *in vitro* and *in vivo*.[36] p,p'-DDE was applied to antagonist testing with AR-CALUX cells to confirm its AR antagonistic activity in our *in vitro* assay. p,p'-DDE was able to inhibit AR-mediated luciferase activity by DHT in a receptor-specific manner (Figure S4). On the basis of the dose−response curve, the weight-based relative potency (REP) compared with that of flutamide was calculated on the basis of the 25% inhibitory concentration (IC_{25}), IC_{50}, and 75% inhibitory concentration (IC_{75}) as 0.72, 0.74, and 0.76, respectively (see Table S3, Supporting Information). To evaluate whether p,p'-DDE was the main contributor to the overall AR antagonistic potency of Baikal seal persistent extracts, the theoretical CALUX-FluEQs of p,p'-DDE were calculated from concentrations (Table S4)[37] and IC_{25}-based REP (Table S3) and compared with the experimental CALUX-FluEQs for individual extracts in a manner similar to that for dioxin-like compounds (described above). p,p'-DDE contributed strongly to the total AR antagonistic activities detected in persistent extracts of Baikal seal blubber (1992, n = 10; 2005, n = 6) and liver (2005, n = 9) (Figure 4). The median theoretical rates of contribution of p,p'-DDE to the experimental CALUX-FluEQs were 64% and 28% for blubber and liver samples, respectively. Although these results suggest that other

AR antagonists might be present in the tested persistent extracts, especially for liver samples, our results indicate that *p,p′*-DDE—the major transformation product of DDT, an internationally banned or severely restricted POP—alone contributed to, and was correlated relatively strongly with, the AR antagonistic activity in Baikal seal persistent extracts (Figure 4). In the former Soviet Union, DDT was used for pest control until the early 1990s, although it was officially banned in 1969/1970 by the Soviet government.[38] The use and production/importation of DDT as a pesticide has been banned since 1971 and 1981, respectively, in Japan. Detection of a DDT transformation product in Baikal seals collected from Lake Baikal but not in common cormorants, raccoon dogs, or finless porpoises collected from Japan, may therefore be attributable to the temporal difference between the two countries in prohibiting the use of DDT. However, other AR antagonists, such as PCBs[29] and persistent pesticides,[26] might also be contributing to AR antagonistic activity in Baikal seal liver samples. In an ongoing study we are investigating the contributions of other compounds to AR antagonistic activity in the livers of a variety of wild animals, using our approach of combining *in vitro* testing with chemical analysis, and we expect to identify other major AR antagonists in the persistent fraction of wildlife in the near future.

Implications of Our Results. Our results demonstrated that combining *in vitro* CALUX assays with instrument analyses of sulfuric acid-treated extracts detected and identified POP-like compounds that were accumulated at high levels in wild animals and had potential endocrine-disrupting effects. Dioxins and *p,p′*-DDE were identified as important target contaminants indicating dioxin-like toxicity and AR antagonistic potency in wildlife top predators. Although previous studies have highlighted the importance of combining *in vitro* bioassays with instrument analyses for identifying potentially toxic compounds in various environmental matrices,[25,35,39−45] our study confirmed the usefulness of this approach as a methodology for determining *in vitro* toxicity profiles.

Our methodology may also be useful for identifying potential contaminants accumulating in wild animals. In this study we applied sulfuric acid-treated extracts to CALUX assay screenings for detecting persistent bioaccumulative endocrine-disrupting chemicals. The rationale for this was that compounds unaffected by highly acidic conditions are likely to be persistent chemicals, such as POPs, which are some of the most problematic compounds found in the environment. In an ongoing study we are evaluating the bioaccumulation of both persistent and acid-labile contaminants in wild animals by fractionating crude extracts untreated with sulfuric acid, on the basis of the compounds' lipophilicity in the CALUX assays and instrument analyses. This will hopefully reveal the important accumulative compounds—including not only POPs but also unidentified contaminants—causing *in vitro* endocrine disruption.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. We provide additional experimental information containing two figures and five tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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