TEMPORAL TRENDS OF DIOXINS AND RELATED COMPOUNDS CONCENTRATIONS AND CYTOCHROME P4501A-DEPENDENT CATALYTIC ACTIVITIES IN COMMON CORMORANTS FROM LAKE BIWA, JAPAN THROUGH 2001 TO 2005

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Abstract
This study presents concentrations of dioxin-like compounds and cytochrome P4501A (CYP1A)-dependent enzymatic activities in common cormorants (*Phalacrocorax carbo*) collected from Lake Biwa, Japan through 2001 to 2005. The hepatic total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalent (TEQ) of polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and coplanar polychlorinated biphenyls was in the range of 180–50,000 pg/g lipid weight. The hepatic alkoxyresorufin *O*-dealkylase (AROD) activities, including methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin *O*-dealkylase activities significantly increased with total TEQ, indicating CYP1A induction by such dioxin-like compounds in the liver of individuals of wild population. With regard to temporal trends, total TEQ in the adult specimens exhibited no significant change during five years investigated. Considering the estimate indicating that dioxin-like compounds released into the environment decreased during the same period due to the national regulation on dioxins emission, the regulation less affects dioxin concentrations in the cormorants at the moment. Furthermore, AROD activities in the hepatic microsomes showed no declining trend, which is consistent with the trend of dioxin concentrations. Our investigation underlines the need for long-term monitoring studies on not only dioxins contamination, but also their potential ecotoxicological risk, focusing on species at the high trophic levels such as cormorant in the ecosystem of Japan.

Introduction
Dioxins and related compounds, including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (Co-PCBs) have been detected in various environmental compartments because of their high persistency and lipophilicity. Such chemical properties contribute to their bioaccumulation and biomagnification through the food web into the high trophic organisms. Many investigations have focused on cormorant, a top predator, widely distributed in aquatic environment as a suitable bioindicator species for understanding contamination and toxic effects of dioxin-like compounds.[1]
The Ministry of the Environment of Japan has announced that emission of dioxins into the environment decreased during the last decade, as a result of national regulation on dioxins emission ("Law Concerning Special Measures against Dioxins" enforced in 2000).[2] However, dioxins released in the past are still deposited in soil and sediment, and the input from such secondary sources may be continuing.[3,4]
A well-characterized biomolecular consequence triggered by dioxin-like compounds is induction of cytochrome P4501A (CYP1A) enzymes mediated by aryl hydrocarbon receptor. It is generally known that the CYP1A induction may be involved in the disruption of the signaling pathway regulated by endogenous substrates, biotransformation to harmful intermediates and production of reactive oxygen species, thus indicating that CYP1A induction by xenobiotics is linked with their toxic effects.
Our recent study reported that common cormorants (*Phalacrocorax carbo*) collected from Lake Biwa, Japan in May 2001 were highly contaminated with dioxin-like compounds, and manifest induction of CYP1A isozymes in the liver.[5-7] We have also suggested that activities of methoxyresorufin *O*-demethylase (MROD), ethoxyresorufin *O*-deethylase (EROD), pentoxyresorufin *O*-depenthylase (PROD) and benzyloxyresorufin *O*-debenzylase (BROD) are all useful for evaluating both exposure to dioxin-like compounds and induction of CYP1A isozymes in the Lake Biwa population of cormorants.[6] However, there is no comprehensive report on temporal variation of dioxin concentrations and CYP1A-dependent activities in wildlife from Japan. It is important to evaluate the effects of dioxins emission control on wild animals, especially organisms at high trophic levels.
The present study aims at clarifying the temporal trends of dioxins accumulation and their effects on CYP1A-dependent enzymatic activities in common cormorants collected from Lake Biwa during 2001–2005.

Particular attention is paid to whether dioxin concentrations and CYP1A-dependent activities decrease in the wild population, reflecting the recent declining trend of dioxins emission.

Materials and Methods

Common cormorants (n = 76) were annually collected from Lake Biwa during 2001–2005 under license. Liver samples were immediately excised after the collection, and the subsamples were flash frozen in liquid nitrogen and stored at –80° C until microsomal preparation. The remaining livers were stored at –20° C until chemical analysis. Growth stage of cormorants was determined from the development of reproductive organs. The numbers of adult specimens captured in 2001, 2002, 2003, 2004 and 2005 were 11, 8, 10, 18 and 9, respectively. The remaining 20 individuals were judged as juveniles (n = 11) or unknown (n = 9).

Chemical analysis of dioxin-like compounds was conducted following the method described previously.[5] Identification and quantification of dioxin-like compounds were performed using a high-resolution gas chromatograph coupled with a high-resolution mass spectrometric detector. Toxic equivalent (TEQ) was calculated using the toxic equivalency factor (TEF) for birds proposed by WHO.[8] MROD, EROD, PROD and BROD activities in the hepatic microsomes of cormorants were measured according to the previously described method.[6]

Results and Discussion

Total TEQ of dioxin-like compounds in the liver of common cormorants captured during 2001–2005 ranged from 9.1 to 1,900 pg/g wet weight (180 to 50,000 pg/g lipid weight) (Table 1). Congener-specific analysis revealed that the contribution from 3,3',4,4',5-P_5CB was the highest, showing 21–62 %. TEQ from 2,3,4,7,8-P_5CDF and 1,2,3,7,8-P_5CDD also contributed greatly to total TEQ, reaching 7.3–26 % and 4.9–14 %, respectively.

Hepatic microsomal AROD activities in cormorants were characterized by the highest activity of EROD (36–770 pg/min/mg protein), followed by MROD (20–440 pg/min/mg protein) or BROD (2.1–300 pg/min/mg protein). PROD activity (1.4–6.3 pg/min/mg protein) was apparently low. Furthermore, there were significant positive correlations (p < 0.0001) between total TEQ and all the AROD activities examined, indicating induction of CYP1A isozyme(s) by the accumulation of dioxins in the liver of cormorants. The relationship between total TEQ and EROD activity is shown in Fig. 1.

To clarify temporal variation of dioxin concentrations, total TEQ in the liver of cormorants collected in each year was compared (Fig. 2). Since TEQ varies greatly depending on the life stage, TEQ from the adult specimens was only subjected to the following statistical analysis.[8] Statistical analyses using Tukey-Kramer multiple comparison test, along with one-factor ANOVA, revealed no significant change in the total TEQ with time. Accordingly, dioxin-like

Table 1 Median and range of TEQ (pg/g wet weight) in the liver of common cormorants collected from Lake Biwa, Japan during 2001-2005.

Congener	Median (range)
2,3,7,8-T_4CDD	2.5 (0.23-19)
1,2,3,7,8-P_5CDD	17 (1.4-180)
1,2,3,4,7,8-H_6CDD	0.26 (0.036-2.2)
1,2,3,6,7,8-H_6CDD	0.14 (0.0089-1.6)
1,2,3,7,8,9-H_6CDD	0.16 (ND-1.3)
1,2,3,4,6,7,8-H_7CDD	0.0084 (ND-0.0070)
1,2,3,4,6,7,8,9-O_8CDD	0.0011 (0.00028-0.017)
2,3,7,8-T_4CDF	0.36 (ND-1.6)
1,2,3,7,8-P_5CDF	0.0092 (ND-0.046)
2,3,4,7,8-P_5CDF	33 (2.4-470)
1,2,3,4,7,8-H_6CDF	0.32 (ND-3.0)
1,2,3,6,7,8-H_6CDF	0.25 (ND-2.8)
1,2,3,7,8,9-H_6CDF	NA
2,3,4,6,7,8-H_6CDF	0.40 (0.041-3.4)
1,2,3,4,6,7,8-H_7CDF	0.0071 (ND-0.048)
1,2,3,4,7,8,9-H_7CDF	NA
1,2,3,4,6,7,8,9-O_8CDF	NA
3,3',4,4'-T_4CB (77)	2.2 (0.68-14)
3,4',4,5'-T_4CB (81)	9.8 (0.79-100)
3,3',4,4',5-P_5CB (126)	53 (2.0-1000)
3,3',4,4,5,5'-H_6CB (169)	0.11 (0.0030-2.1)
2,3,3',4,4'-P_5CB (105)	2.0 (0.096-27)
2,3,4,4',5-P_5CB (114)	0.15 (0.0066-2.3)
2,3',4,4',5-P_5CB (118)	0.64 (0.0019-9.4)
2',3,4,4',5-P_5CB (123)	0.012 (0.00050-0.21)
2,3,3',4,4',5-H_6CB (156)	1.0 (0.027-17)
2,3,3',4,4',5'-H_6CB (157)	0.23 (0.0072-3.6)
2,3',4,4',5,5'-H_6CB (167)	0.050 (0.0012-0.86)
2,3,3',4,4',5,5'-H_7CB (189)	0.010 (ND-0.16)
Total TEQ	130 (9.1-1900)

NA and ND denote no data available and not detected, respectively.

compounds that accumulate in the cormorant livers appear not to decrease through 2001 to 2005. In consistent with our results, Kunisue et al. (2006) showed that total TEQ in Japanese human breast milk from primiparae have not decreased from 1998 to 2004.[9] On the other hand, an estimate from the national survey on dioxins emission inventory has shown a declining trend of dioxins emission from 1899-2013 g-TEQ/y in 2001 to 323-348 g-TEQ/y in 2005 (Fig. 2).[2] These results clearly indicate that dioxin concentrations in wildlife and human may not decrease as dioxins emission rapidly decreased following the national regulation. Apart from this, there is no information on the data of dioxins emission around Lake Biwa, and thus, it remains unclear whether dioxins emission in this region has decreased periodically through 2001 to 2005.

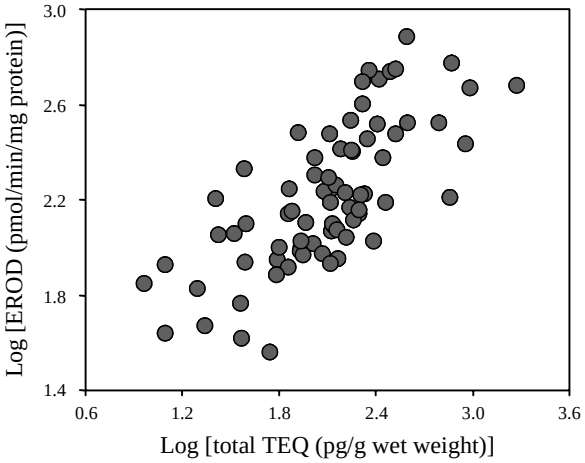

Fig. 1. Relationship between total TEQ and EROD activity in the liver of common cormorants. $P < 0.0001$.

Annual data of each AROD activity in adult common cormorants were also statistically analyzed (Fig. 3). The results represented that there is no consistent temporal trend of each AROD activity, although some annual variations were statistically detected. Our recent study addressing the catalytic function of cormorant CYP1A4 and 1A5 that were heterologously expressed using an *in vitro* system have revealed that MROD and BROD activities are specifically catalyzed by CYP1A5 and CYP1A4, respectively, whereas EROD activity is catalyzed in a similar efficiency by both CYP1A isozymes.[10] The annual data of each AROD activity indicate that there is little sign of declining trends in these enzymatic activities, and thus CYP1A4 and 1A5 are chronically induced in the liver of wild cormorants.

These observations suggest that long-term monitoring studies of dioxins contamination in wild species at high tropic levels are necessary even after the regulation on dioxins emission was implemented. The present study also indicates that measurement of AROD activity may be a cost-effective and easy-to-use approach to monitor the accumulation of dioxin-like compounds and expression of CYP1A isozymes in wildlife.

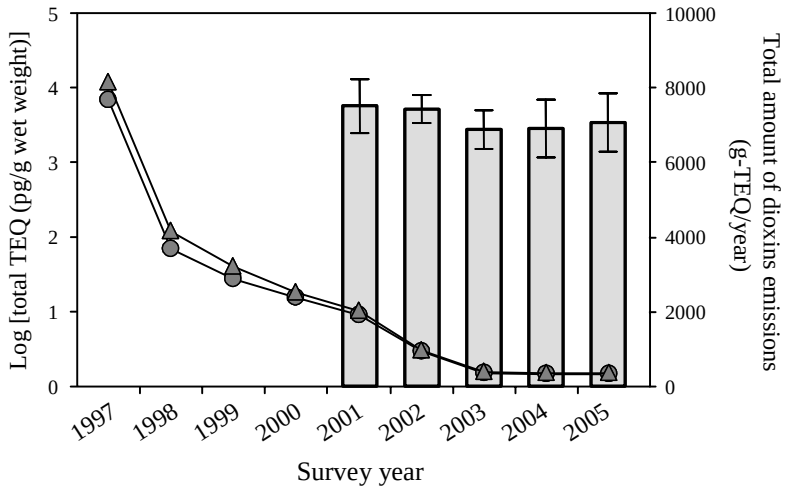

Fig. 2. Temporal trends of total TEQ in the liver of common cormorants from Lake Biwa and estimates of dioxins emission in Japan. Bars show total TEQ (mean±standard deviation) in the liver of cormorants. Dioxins emission is represented as line plots of minimum (circle) and maximum (triangle). Data on national dioxins emissions inventory are cited from the Ministry of the Environment, Japan (2005).

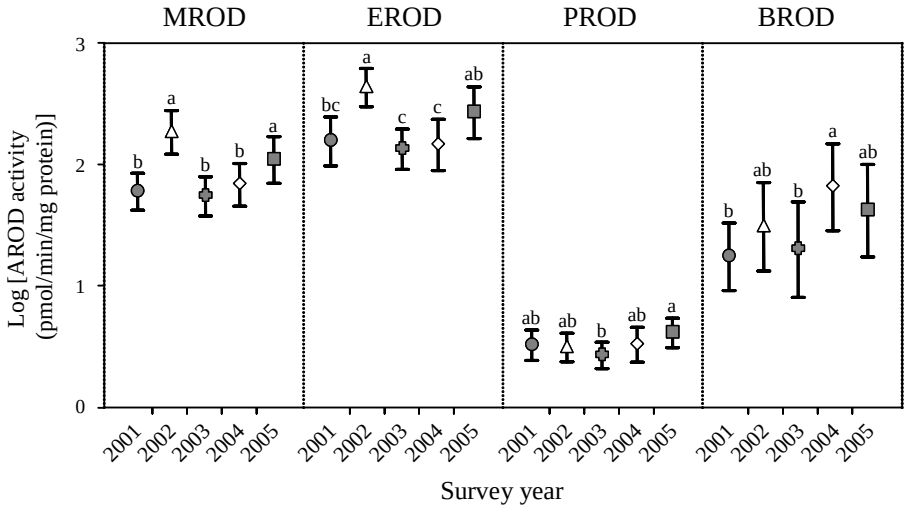

Fig. 3. Temporal trends of hepatic microsomal AROD activities in common cormorants during 2001-2005. AROD activities with different letters are significantly different at $p < 0.05$. Each point and bar represents the mean and standard deviation, respectively.

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