PCDD/DFs AND DIOXIN-LIKE PCBs IN FISH OIL USED FOR FEED INGREDIENTS IN JAPAN: EVALUATION BY 2005 WHO-TEF AND CALUX ASSAY

Hasegawa J[1], Guruge KS[1], Seike N[2], Shirai Y[3], Yamata T[3], Nakamura M[4], Handa H[4], Yamanaka N[1], Miyazaki S[1]

[1]National Institute of Animal Health, Kannondai 3-1-5, Tsukuba, Ibaraki, 305-0856, Japan
[2]National Institute for Agro-Environmental Sciences, Kannondai 3-1-3, Tsukuba, Ibaraki 305-0856, Japan
[3]Food and Agricultural Materials Inspection Center, Shintoshin 2-1, Chuo-ku, Saitama, Saitama 330-9733, Japan
[4]Hiyoshi Ecological Services Co., Kitanoshyo 908, Omihachiman, Shiga 523-0806, Japan

Abstract
Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/DFs) and dioxin-like polychlorinated biphenyls (DL-PCBs) in 41 fish oil samples used for feed ingredients in Japan were determined by using HRGC/HRMS and CALUX bioassay. The mean TEQ values derived from conventional 1998 WHO-TEF of PCDD/DFs and DL-PCBs were 2.6 and 9.9 pg/g ww, respectively. The levels of TEQ derived from the recently re-evaluated 2005 WHO-TEF were slightly lower than those of the former in both groups. Notably, the contribution of mono-ortho DL-PCBs to total 2005 WHO-TEQ was considerably decreased compared to the case of 1998 WHO-TEQ, resulting from the reduction in its TEF values, while the non-ortho DL-PCBs contribution was increased. The mean TEQ determined by CALUX assay for PCDD/DFs was approximately three times higher, whereas DL-PCBs was approximately two times lower than WHO-TEQ determined by HRGC/HRMS; the sum of PCDD/DFs and DL-PCBs was very similar by both methods. The correlation coefficients between the CALUX-TEQ and 2005 WHO-TEQ were 0.82, 0.89, and 0.90 for PCDD/DFs, DL-PCBs, and the sum, respectively. These results suggest that the CALUX assay is substantially useful method for the screening of dioxin-related compounds in fish oils.

Introduction
Fish oils are known sources of nutritionally valuable components such as polyunsaturated fatty acids. The crude fish oil products are used as feed ingredients for farmed fish and domestic animals, however it has been reported that fish oils contain relatively high levels of PCDD/DFs, and DL-PCBs compared to other feed ingredients [1, 2]. It is important to routinely monitor these compounds in a vast number of samples to avoid producing feeds with elevated levels of contamination. The regular method for dioxin analysis using HRGC/HRMS would be costly and time consuming to cover up large number of samples. Meanwhile, the CALUX assay has been widely employed as a rapid analysis of dioxin-like toxicity in environmental and biological samples including food and feed [3-5]. And while the TEF proposed by WHO (WHO-TEF) [6] was re-evaluated in recent years [7], it has been little described about not only the results of change of TEQ levels and contributions in substantial samples but also the correlation between the re-evaluated TEQ based on chemical analysis and TEQ based on bioanalytial method such as CALUX assay. We previously carried out the CALUX assay for fish oils used as feed ingredients in Japan, but the number of sample was relatively limited [8]. In this study, we expanded the number and collecting year of samples for analysis, and the contamination levels of PCDD/DFs and DL-PCBs were determined by chemical analysis using HRGC/HRMS and CALUX assay. In the chemical analysis, TEQ were calculated using the two WHO-TEFs, and the difference in results was noted. TEQ of PCDD/DFs and DL-PCBs obtained by the CALUX assay were compared with WHO-TEQ.

Materials and Methods
Sample collection
A total of 41 fish oil products were collected from several feed manufactories in Japan during 2000-05. These samples were stored at -20℃ until analysis.

Chemical analysis
PCDD/DFs and DL-PCBs were analyzed according to the provisional guideline for analytical method of dioxin related compounds in feed described by the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan [9]. A

10 g of fish oil sample was spiked with ^{13}C-labeled 2,3,7,8-substituted PCDD/DFs and DL-PCBs as internal standards, and subjected to KOH/EtOH digestion at room temperature for two hours. Then the alkaline solution was diluted with distilled water, and extracted with hexane three times. The extract was cleaned up by the multi-layer silica gel column, followed by separated to mono-ortho DL-PCBs fraction and non-ortho DL-PCBs and PCDD/DFs fraction using the activated carbon column. The fractionated extracts were concentrated and spiked some congeners of ^{13}C-labeled PCDD/DFs and PCBs as recovery standards. Determination of PCDD/DFs and DL-PCBs were performed by HRGC/HRMS. The average recovery of PCDD/DFs and DL-PCBs were in the range of 50-120%. The limit of quantification (LOQ) of PCDD/DFs, non-ortho DL-PCBs and mono-ortho DL-PCBs were 0.05-0.2 pg/g, 0.1 pg/g and 0.5 pg/g wet weight (ww) basis, respectively. TEQ were calculated using the both conventional 1998 and re-evaluated 2005 WHO-TEFs (Table 1). In case where the congeners were below those of LOQ, TEQ were calculated as lower bound values, which are equal to zero.

CALUX assay

The assay was carried out as previously described [8]. A 1.0-1.3 g of fish oil sample dissolved in hexane was mixed with about 17 g of 44% (w/w) sulfuric acid-silica gel and shaken followed by left stand for 30 minute. The mixture was passed through the sulfuric acid-silica gel column and eluted with hexane. The eluent was loaded on XCARB carbon (Xenobiotic Detection Systems Inc.) column. The column was washed with hexane to remove remaining interfering matrix and the bulk of 2-4 ortho substituted PCBs. Then DL-PCBs fraction was eluted with 15 ml of hexane/toluene/ethyl acetate (8:1:1), followed by PCDD/DFs fraction was eluted with 20 ml of toluene. The fractionated extracts were evaporated and resuspended in hexane. Aliquots of the hexane solution were reconstituted with 4 µl of DMSO. Immediately prior to dosing cells, the sample extracts and 2,3,7,8-TCDD standard solutions (4 µl of DMSO) was diluted in 400 µl of culture medium. H1L6.1 mouse hepatoma cells were cultured in 96-well microplate at 7.5 x 10^5 cell/well. After 24 hours incubation, cells were exposed in duplicate to the purified sample extracts and the 9 serial dilutions of 2,3,7,8-TCDD standard as well as DMSO alone on each plate. Following 20-24 hours of incubation, the luciferase activity was measured using luminometer. Results were expressed in relative light unit (RLU) and the average RLU value of DMSO alone was subtracted from all RLU values. CALUX-TEQ was calculated according to standard curves. Calculated LOQ of CALUX-TEQ in the sample extracts depended on sample volume and dilution factor was 1.44-1.97 pg/g ww for PCDD/DFs and 0.868-1.00 pg/g ww for DL-PCBs. Relative potency (REP) values [10] for individual congeners of PCDD/DFs and DL-PCBs were shown in Table 1.

Table 1. WHO-TEFs and CALUX-REP values.

congener	WHO-TEF 1998 [a]	WHO-TEF 2005 [b]	CALUX-REP [c]
PCDD/DFs			
2,3,7,8-TeCDD	1	1	1
1,2,3,7,8-PeCDD	1	1	0.73
1,2,3,4,7,8-HxCDD	0.1	0.1	0.075
1,2,3,6,7,8-HxCDD	0.1	0.1	0.098
1,2,3,7,8,9-HxCDD	0.1	0.1	0.061
1,2,3,4,6,7,8-HpCDD	0.01	0.01	0.031
OCDD	0.0001	0.0003	0.00034
2,3,7,8-TeCDF	0.1	0.1	0.067
1,2,3,7,8-PeCDF	0.05	0.03	0.14
2,3,4,7,8-PeCDF	0.5	0.3	0.58
1,2,3,4,7,8-HxCDF	0.1	0.1	0.13
1,2,3,6,7,8-HxCDF	0.1	0.1	0.14
1,2,3,7,8,9-HxCDF	0.1	0.1	0.11
2,3,4,6,7,8-HxCDF	0.1	0.1	0.31
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.024
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.044
OCDF	0.0001	0.0003	0.0016
DL-PCBs			
Non-ortho PCBs			
PCB 77	0.0001	0.0001	0.0014
PCB 81	0.0001	0.0003	0.0045
PCB 126	0.1	0.1	0.038
PCB 169	0.01	0.03	0.0011
Mono-ortho PCBs			
PCB 105	0.0001	0.00003	0.000001
PCB 114	0.0005	0.00003	0.00014
PCB 118	0.0001	0.00003	0.000001
PCB 123	0.0001	0.00003	0.0000003
PCB 156	0.0005	0.00003	0.00014
PCB 157	0.0005	0.00003	0.000003
PCB 167	0.00001	0.00003	0.0000003
PCB 189	0.0001	0.00003	0.0000002

[a,b] WHO-TEF were cited from Van den Berg et al. (1998, 2006)
[c] CALUX-REP were cited from Brown et al. (2001) and provided by XDS Inc.

Results and Discussion

Evaluation by two WHO-TEFs derived from chemical analysis

Mean TEQ calculated by 1998 WHO-TEF were 2.6, 9.9, and 12.5 pg/g wet weight (ww) for PCDD/DFs, DL-PCBs, and the sum, respectively. The mean and range of contribution of DL-PCBs to total TEQ was 79 (63-91) %. It has been reported that the changes in 2005-TEF have a limited impact on the total TEQ of some biological samples with overall decrease in TEQ ranging between 10 and 25% [7]. TEQ calculated by re-evaluated

2005 WHO-TEF in fish oils were somewhat less than the results from conventional 1998-TEF. The mean and range of the ratio of 2005 to 1998-TEQ in fish oil samples analyzed in this study were 0.83 (0.71-0.98) for PCDD/DFs, 0.89 (0.81-1.06) for DL-PCBs, and 0.89 (0.80-1.00) for the sum.

The contribution of PCDFs to total 2005-TEQ was marginally decreased, and those of mono-ortho DL-PCBs were considerably decreased from 15 to 4% (1.9 to 0.4 pg/g ww in actual TEQ) compared to 1998-TEQ. These results are the outcome of the reduction in TEF values for 2,3,4,7,8-PeCDF and all the mono-ortho DL-PCB congeners except for PCB 167 in 2005-TEF. In contrast, the non-ortho DL-PCBs contribution was increased from 64 to 76% (8.0 to 8.4 pg/g ww in actual TEQ). These results suggest that non-ortho DL-PCBs are substantially important target with regard to evaluating the risk of dioxin-like compounds in fish-originated feed and food samples. Among all congeners, the highest contributor to total TEQ derived from both TEFs was PCB 126 in all samples, and the proportion was 63 (52-79) % for 1998-TEQ and 71 (59-80) % for 2005-TEQ. Although TEQ levels were measurably decreased in most biological samples as a result of changing TEF, the presence of other polyhalogenated aromatic compounds with possible AhR-activity should be taken into account.

Evaluation by CALUX assay and comparison with WHO-TEQ

Figure 1 shows the result of comparing CALUX-TEQ obtained from a PCDD/DFs fraction and DL-PCBs fraction with both 1998 and 2005 WHO-TEQ measured by chemical analysis. The mean CALUX-TEQ for PCDD/DFs was approximately three times higher, while DL-PCBs was approximately two times lower than those of WHO-TEQ. Such a tendency has been observed in other investigations that have analyzed fish-originated samples [11-14]. Meanwhile, CALUX-TEQ obtained from the sum of PCDD/DFs and DL-PCBs was very close to WHO-TEQ because the overestimation for PCDD/DFs was compensated by the underestimation for DL-PCBs in the CALUX assay.

To examine the factors affecting the discrepancy found in TEQ measured by bioassay and chemical analysis, REP-TEQ (theoretical CALUX-TEQ) were calculated by multiplying the concentration of each congener determined by HRGC/HRMS by its CALUX-REP value instead of WHO-TEF, and compared to WHO-TEQ and CALUX-TEQ (Fig. 1). REP-TEQ of PCDD/DFs was found to be extremely close to WHO-TEQ; it is therefore speculated that the presence of other AhR agonists, not but the difference between WHO-TEF and CALUX-REP is responsible for the discrepancy. On the other hand, REP-TEQ of DL-PCBs was lower than WHO-TEQ and similar to CALUX-TEQ, thus the discrepancy in the two methods was probably due to the difference between WHO-TEF and CALUX-REP. The REP value of PCB 126, which accounted for 79 (70-89) % of total 1998 WHO-TEQ in all samples, is 2.6 times less than WHO-TEF, resulting in approximately two times lower CALUX-TEQ of DL-PCBs than WHO-TEQ.

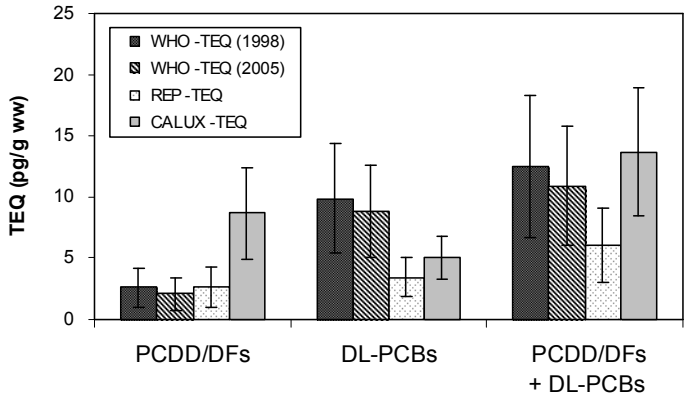

Fig. 1. Comparison of mean TEQ levels in 41 fish oil samples obtained by HRGC/HRMS analysis and CALUX assay. WHO-TEQ and REP-TEQ were calculated using 1998 and 2005 WHO-TEF, and CALUX-REP instead of WHO-TEF, respectively. Error bars are standard deviations.

Relatively good relationships were observed in TEQ determined by the CALUX assay and HRGC/HRMS analysis (Fig. 2). The correlation coefficients between CALUX-TEQ and 2005 WHO-TEQ were 0.82, 0.89, and 0.90 for PCDD/DFs, DL-PCBs and the sum, respectively. The same result was obtained in 1998 WHO-TEQ, except for the 0.84 of PCDD/DFs. In the case of the sum of PCDD/DFs and DL-PCBs, WHO-TEQ could be predicted from CALUX-TEQ, as the slope value of linear regression equation for the two methods is nearly 1 (y=x). Although care must be taken when the results of the two methods are directly compared, the CALUX assay in combination with reference chemical analysis could help to control the safety in feed production and circulation.

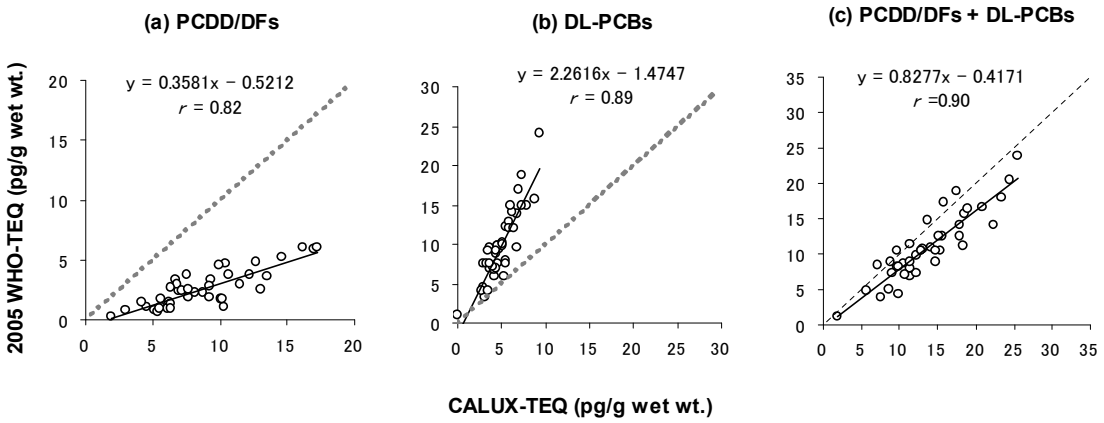

Fig. 2. Correlation between CALUX-TEQ and 2005 WHO-TEQ determined by HRGC/HRMS analysis in 41 fish oil samples. CALUX-TEQ of (a), (b), (c) were obtained from PCDD/DFs fraction, DL-PCBs fraction, and sum of the result of (a) and (b), respectively. Dashed line indicates the bisecting linear correlation (y=x).

Acknowledgements
This work was supported in part by a Grant-in-aid under the Research project for utilizing advance technologies in agriculture, forestry, and fisheries in Japan.

References

1. Gallani B, Verstraete F, Boix A, von Holst C, Anklam E. *Organohalogen Compd*. 2004; 66:1893-1900.
2. Guruge KS, Seike N, Yamanaka N, Miyazaki S. *Chemosphere*. 2005; 58:883-9.
3. Behnisch PA, Hosoe K, Sakai S. *Environ. Int*. 2001; 27:495-519.
4. Hoogenboom L, Goeyens L, Carbonnelle S, van Loco J, Beernaert H, Baeyens W, Traag W, Bovee T, Jacobs G, Schoeters G. *Trends. Anal. Chem*. 2006; 25:410-20.
5. Scippo M-L, Rybertt S, Eppe G, Massart A-C, de Pauw E, Maghuin-Rogiste, G. *Accred. Qual. Assur*. 2006; 11:38-43.
6. Van den Berg M, Birnbaum L, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T. *Environ. Health Perspect*. 1998; 106:775-92.
7. Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson, RE. *Toxicol. Sci*. 2006; 93:223-41.
8. Guruge KS, Shirai Y, Yamata T, Morito K, Nakamura M, Yamanaka N, Miyazaki S. *Organohalogen Compd*. 2006; 68:1885-8.
9. MAFF of Japan. *The provisional guideline for analytical method of dioxin related compounds in feed*. 2004.
10. Brown DJ, Chu M, van Overmeire I, Chu A, Clark GC. *Organohalogen Compd*. 2001; 53, 211-4.
11. Besselink H, Jonas A, Pijnappls M, Swinkels A, Brouwer B. *Organohalogen Compd*. 2003; 60: 203-6.
12. Tsutsumi T, Amakura Y, Nakamura M, Brown DJ, Clark GC, Sasaki K, Toyoda M, Maitani T. *Analyst*. 2003;128:486-92.
13. Carbonnelle S, van Loco J, van Cleuvenbergen R, van Leeuwen S, van Overmeire I, Windal I, van Wouwe N, Goeyens L. *Organohalogen Compd*. 2004; 66:336-40.
14. Windal I, van Wouwe N, Eppe G, Xhrouet C, Debacker V, Baeyens W, de Pauw E, Goeyens L. *Environ. Sci. Technol*. 2005; 39:1741-8.