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Application of Bioassays for the Detection of Dioxins and Dioxin-like Compounds in Wastes and the Environment

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Abstract—Dioxin bioassays are gaining widespread use and regulatory acceptance by developing appropriate sample cleanups and validating with high-resolution instrumental analysis. Cell-based aryl hydrocarbon receptor (AhR)/reporter gene assays are sensitive to polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), and their bioassay-toxic equivalent factors for PCDD/Fs were close to World Health Organization (WHO)-equivalent factors, which yields a clear linear relationship between bioassay toxic equivalents and WHO-toxic equivalents in various environmental media. In addition, other than PCDD/Fs, unknown chemicals such as brominated or mix-halogenated aromatic hydrocarbons may exert similar dioxin-like toxic effects through AhR activation. Thus, AhR/reporter gene assay can be a promising tool to measure the comprehensive effects of dioxin-like compounds and to identify responsible compounds combined with chemical analysis. In this paper, our case study results conducted for Toxicity Reduction Evaluation and Toxicity Identification Evaluation approaches using Dioxin Responsive-Chemical Activated LUciferase eXpression (DR-CALUX) assay were briefly summarized.

Keywords: dioxin-like compounds, bioassays, AhR/reporter gene assay, waste, environment, Toxicity Reduction Evaluation, Toxicity Identification Evaluation

INTRODUCTION

Over the past decade, development, application and validation of bioanalytical methods (bioassays) for the detection and relative quantification of dioxins and related compounds (i.e., dioxin-like compounds) have been intensively conducted (Behnisch *et al*., 2001; Sakai and Takigami, 2003). Especially, cell-based aryl hydrocarbon receptor (AhR)/reporter gene assays and kit-based immunoassays have been developed in combination with appropriate sample cleanups, which can be coupled with complementary high-resolution mass spectrometry instrumental analysis. These methods are gaining widespread use and regulatory acceptance (the Commission of the European Communities, 2002; Nakano *et al*.,

Table 1. Official methods for dioxin measurement in wastes (notified by the Ministry of the Environment of Japan, Sep. 2005)

No.	Categories	Cleanup methods	Biological materials
1-1	AhR reporter gene assay	Sulfuric acid/silica gel column + carbon column	Mouse recombinant cell (H1L6. 1c2) CALUX
1-2		Sulfuric acid/silica gel column + carbon column	Human recombinant cell (101L) P450 HRGS
1-3		Multilayer silica gel column	Mouse recombinant cell (HeB5) Sumitomo
2	Immunoassay	Multilayer silica gel + carbon column	Anti-dioxin monoclonal antibody (specific to PeCDD/Fs) Dio-quicker

2006; US EPA, 2008).

In this paper, official approval and use of the dioxin bioassays worldwide were briefly reviewed. In the context of current international use of bioassays, they are alternative analytical methods to instrumental analysis for polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), and dioxin-like polychlorinated biphenyls (dl-PCBs). However, the bioassays can provide important information on the overall dioxin-like potency of complex mixtures, which enables to conduct two important approaches, namely Toxicity Reduction Evaluation (TRE) and Toxicity Identification Evaluation (TIE). Here we give an overview about our case studies with the Dioxin Responsive-Chemical Activated LUciferase eXpression (DR-CALUX) assay using the rat hepatoma H4IIE recombinant cell line (Murk *et al.*, 1996) for TRE during waste treatment processes and TIE for environmental samples.

OFFICIAL USE OF THE DIOXIN BIOASSAYS

Internationally, the US EPA accepted Method 4425 (human cell-based P450HRGS assay), Method 4025 (immunoassay using a polyclonal antibody specific for PCDD/Fs) and Method 4435 (mouse cell-based CALUX assay) into the SW-846 Compendium of Solid Waste for the screening of dioxins in the specific media in 2000, 2002 and 2007, respectively (US EPA, 2008). For a screening purpose of dioxins in food and feedstuffs in EU, cell-based AhR/reporter gene assay and immunoassay, which fulfills quality assurance/quality control requirements, have been recognized as official methods in 2002 (the Commission of the European Communities, 2002). In 2005 in Japan, the Government allowed the use of bioassay methods (three AhR/reporter gene assays and one immunoassay, see Table 1), which gave acceptable results through official evaluation, for measuring dioxins in emission gas from small scale waste incinerators and incineration ash from all the waste incinerators (Nakano *et al.*, 2006). Also in Japan, immunoassay technologies have been demonstrated to screen residual PCBs in stockpile transformer oil samples even at 0.5 mg/kg

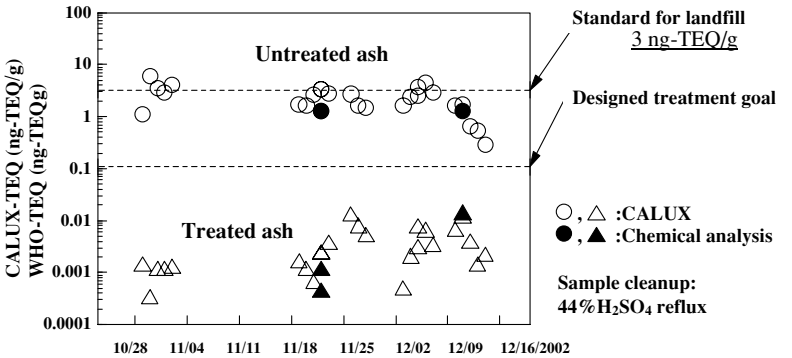

Fig. 1. Dioxin monitoring results during the Hagenmaier process at one actual plant using DR-CALUX and chemical analysis.

which is the stringent PCB treatment standard for national PCB waste treatment (Ohmura *et al*., 2007). The availability of such bioassay methods will greatly facilitate many large-scale screening/monitoring works where the equipment and/or fund are limited. Field surveys with the bioassays have been frequently conducted to overview the pollution status in the concerned media or specify hot spots, taking advantage of "on-site" analysis by bioassays.

TOXICITY REDUCTION EVALUATION (TRE) APPROACH USING THE DIOXIN BIOASSAY

Bioassay analysis can be a promising tool to measure the reduction of dioxins and dioxin-like compounds comprehensively. The case studies described here show an application of DR-CALUX as a TRE tool during waste treatment processes.

DR-CALUX monitoring of dioxins and dioxin-like compounds in fly ash during low temperature thermal dechlorination treatment process

Hagenmaier process has been used at over sixty facilities in Japan to reduce the amount of dioxins and halogenated compounds in fly ash. This process is based on the dechlorination of dioxins under low-temperature and oxygen-deficient reductive conditions (Behnisch *et al*., 2002). We conducted an everyday monitoring with DR-CALUX at one actual plant for two months (Matuyama *et al*., 2003). Raw or treated fly ash samples were pretreated with hydrochloric acid and extracted with toluene in a Soxhlet extraction apparatus for 16 h. The toluene extracts were concentrated, replaced with *n*-hexane and cleaned up by the reflux treatment with 44% sulfuric acid/silica gel. The reflux fraction was then evaporated and redissolved in dimethysulfoxide (DMSO), which was applied to DR-CALUX. The time for monitoring from sampling to the end of DR-CALUX analysis was just four days. As a result, it was confirmed CALUX-TEQ showing several

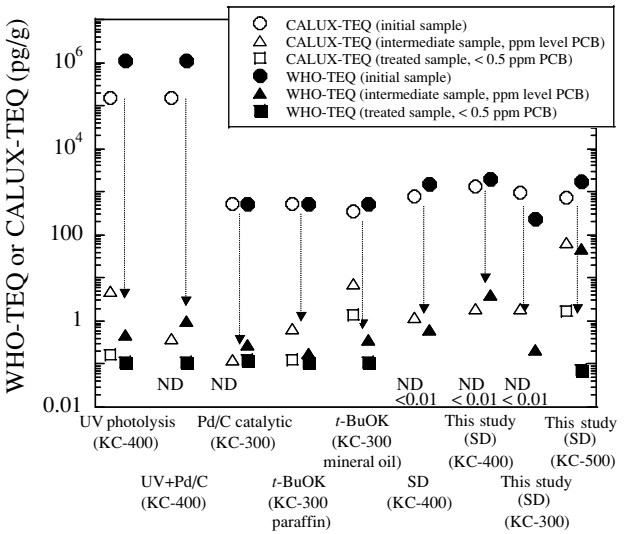

Fig. 2. Comparison of CALUX-TEQ and WHO-TEQ values of PCB (treated) samples during various chemical treatments.

ng/g in untreated ash decreased by two orders of magnitude or more after treatment, in which over 99% treatment efficiency was obtained (Fig. 1). Furthermore, the values were in accordance with World Health Organization-toxic equivalents (WHO-TEQ) values for PCDD/Fs and dl-PCBs measured in between a series of bioassay analysis.

DR-CALUX monitoring of dioxin-like compounds during chemical treatment processes of PCB stockpile

In view of increasing risk by waste PCB stockpiles, the Government of Japan started regional treatments to destroy PCBs stored across the nation from 2004 (Kimura, 2007). For PCB containing oil treatment, which is of major concern, well-demonstrated chemical treatment technologies were adopted. Oil treatment goal standard for PCB has been set as 0.5 ppm. Additionally, focus is also put on the control and reduction of dioxins, hydroxylated PCBs, etc. Especially, monitoring of the fate of dioxin-like compounds during treatment is one of the important toxicological focuses. Figure 2 shows the reduction of CALUX-TEQ (white plot) and WHO-TEQ (black plot) values of PCB oil samples during UV photolysis, Pd/C catalytic hydrodechlorination, potassium *t*-butyloxide dechlorination and sodium dispersion methods (Takigami *et al*., 2004). Here the reflux method with 44% silica gel-sulfuric acid was adopted for the clean-up and fractionation of stable compounds (i.e., dioxin-like compounds) in samples. All of the investigated chemical PCB treatment methods reduced the WHO-TEQ up to 10^{-1} pg/g order or less in the final treated oil samples. The corresponding

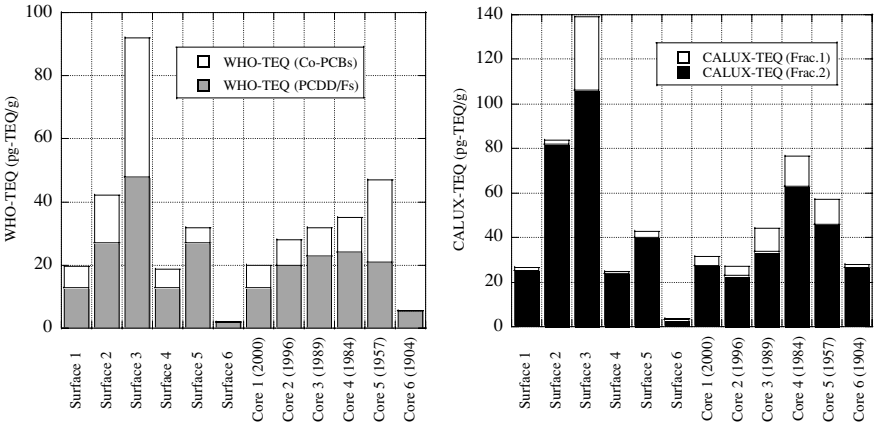

Fig. 3. WHO-TEQ (left) and CALUX-TEQ (right) values in surface and core sediments from Osaka Bay.

CALUX-TEQs showed 1 pg/g or less and CALUX- and chemical TEQs were in consistency. The residual TEQ levels could be satisfactorily low even if compared to strict dioxin limit values of feed and food in EU.

TOXICITY IDENTIFICATION EVALUATION (TIE) APPROACH USING THE DIOXIN BIOASSAY

Other than PCDD/Fs and dioxin-like PCBs, unknown chemicals (e.g., brominated or mixed-halogenated compounds) may exert similar dioxin-like toxic effects through AhR activation, etc. Another powerful strategy for the bioassay is applying a tiered approach with the aim to identify responsible substances combined with chemical analysis. Our two case studies adopting TIE approach were abstracted as shown below (Takigami *et al*., 2005; Suzuki *et al*., 2007a).

Bio/chemical analysis of dioxin-like compounds in sediment samples

The combinatorial bio/chemical investigation of sediments from Osaka Bay, Japan was conducted to clarify the horizontal and vertical distribution profiles of dioxin-like compounds in the sediments (Fig. 3) (Takigami *et al*., 2005). For surface sediments, WHO-TEQ values ranged from 1.8 to 92 pg/g dry weight and CALUX-TEQ values (3.7–140 pg/g dry weight) yielded significant correlation with them ($r^2 = 0.96$). On the other hand, the correlation between both TEQs (for WHO-TEQ, 5.5–47 pg/g dry weight and for CALUX-TEQ, 27–76 pg/g dry weight) for core samples was not significant ($r^2 = 0.46$). Comparing the vertical profiles of CALUX-TEQ and WHO-TEQ, they were different in that WHO-TEQ reached the maximum in the 1957 core section, while CALUX-TEQ reached in the 1984 core section. CALUX-TEQ values were 1 to 5-fold higher than WHO-

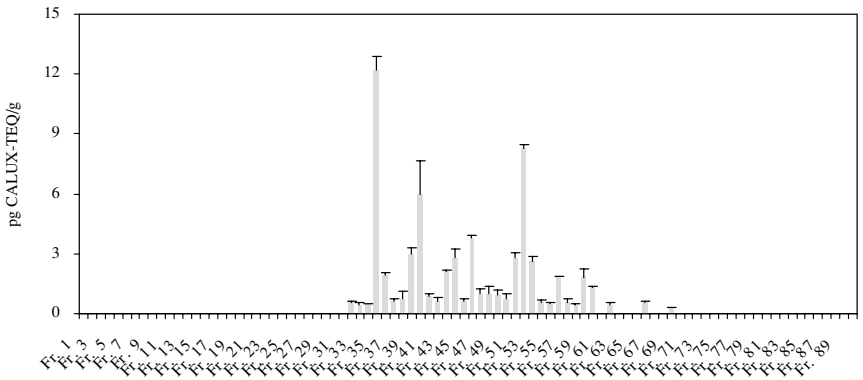

Fig. 4. The dioxin-like activity profile of the separated ODS-HPLC fractions derived from NITRO-HPLC 1st fraction of mixed housed dust.

TEQ values in all the surface and core samples. CALUX-TEQ values were calculated theoretically for polybrominated diphenylethers and polybrominated dibenzo-*p*-dioxins and dibenzofurans (PBDD/Fs), employing their CALUX toxicity equivalent factors (CALUX-TEFs) (Behnisch *et al.*, 2003). The estimated CALUX-TEQ values obtained for the brominated compounds could explain for 11% on average (range 4.7–31%) of the experimentally obtained CALUX-TEQ values in the investigated surface sediments.

Identification of dioxin-like compounds in house dust samples

We have reported relatively higher dioxin-like activity levels in indoor dust samples collected from Japan than those in contaminated sediments (Suzuki *et al.*, 2007b). Exposure assessment indicated that the average daily dose (ADD) of dioxin-like compounds via house dust is comparable to ADDs of dioxins via food. Therefore, dust is a significant exposure pathway to children for dioxin-like compounds. We conducted chemical fractionation of house dust extracts (sulfuric acid treatment extracts) for the purpose of identifying dioxin-like compounds using High Performance Liquid Chromatography (HPLC) and DR-CALUX (Suzuki *et al.*, 2007a). An extract mixture from seven house dust samples was fractionated using normal phase-HPLC fractionation using a nitrophenylpropylsilica column (NITRO-HPLC). All the NITRO-HPLC fractions were assessed for dioxin-like activity using DR-CALUX. The 1st fraction possessed highest dioxin-like activity. This fraction was further fractionated using reverse phase-HPLC fractionations using an octadecylsilica column (ODS-HPLC). All the 90 fractions were tested using the DR-CALUX (Fig. 4). Taking the elution times of the standard compounds into account, the dioxin-like compounds in ODS-HPLC fractions showing high activity were estimated to be the congeners of PCBs, polychlorinated naphthalenes (PCNs), and PBDD/Fs. Tentative Selected Ion Monitoring (SIM) scan using gas chromatography/high-

resolution mass spectrometry for ODS-HPLC fractions showed that PentaCBs, HeptaCBs, TetraCNs, PentaCNs, HeptaCNs, and TetraBDDs were identified and confirmed to contribute to a significant part of the whole activity in the indoor dust.

CONCLUSIVE REMARKS

It should be noted that attention has been increasingly paid to the effective use of *in vitro* bioassay testing and monitoring. Not limited to PCDD/Fs and dioxin-like compounds, bioassays have been expected to tackle problems concerning the cost and time required for *in vivo* toxicity testing, increasing necessity for the evaluation of a number of synthetic chemicals and animal welfare issues. Furthermore, the application of bioassays to the environment and emission source monitoring can be very powerful in order to identify and control the toxic compounds concerned (i.e., for the purpose of TIE and TRE) with case-by-case validations of the bioassays.

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