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Fast Cleanup System Combined with a Dioxin-responsive Element-driven Luciferase Bioassay for Analysis of Polychlorinated Dibenzo-*p*-dioxins/furans in Sediments and Soils

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Abstract A green technique was designed for assessing toxic equivalence (TEQ) levels of polychlorinated dibenzo-p-dioxins/furans (PCDD/Fs) in sediments and soil from dioxin-contaminated areas. This technique combines a fast cleanup system with a dioxin-responsive-element (DRE)-driven luciferase bioassay. Samples from sediment (n = 10) and soil (n = 11) were analyzed with the technique; levels of PCDD/Fs ranged from 75.1 to 2,670 ng DRE-driven luciferase activity (DL)-TEQ/kg dry weight (d.w.). Significant correlations were found between DL-TEQs (by the bioassay) and PCDD/F WHO-TEQs (by HRGC/HRMS). DL-TEQs is significantly correlated with WHO-TEQs of 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD using a multiple stepwise linear regression model (adjusted $R^2 = 0.962$, $p < 0.001$).

Keywords PCDD/Fs · Dioxins · Luciferase · Bioassay

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Polychlorinated dibenzo-*p*-dioxins (PCDDs) and furans (PCDFs) are halogenated aromatic hydrocarbons that have been considered as environmental endocrine disruptors. These organochlorines raise public concern because exposure to dioxins has been associated with several adverse health effects on humans (Van den Berg et al. 2006). Although the multicolumn (silica, alumina, and carbon columns) cleanup procedure followed by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) is widely used in PCDD/Fs analysis, this HRGC/HRMS method has several disadvantages, including cost and high consumption of time and energy (Hoogenboom et al. 2006). To improve the efficiency of PCDD/F measurements of the environmental samples, a fast, green, cheap, and effective cleanup system (Soxtherm with a CAPE-coupled carbon-acid silica column) has been established by a research team with the Environmental Analysis Laboratory (EAL) of Taiwan's Environmental Protection Administration (EPA) (Chen et al. 2007, Lee et al. 2009). The quality assurance/quality control (QA/QC) of this green cleanup system meets the criteria of US EPA methods 1613B, 1668A, and 1614, as well as of the Taiwanese EPA Method (Lee et al. 2009).

The chemically activated luciferase gene express (CALUX) bioassay has been popularly used to detect CALUX-toxic equivalency (TEQ) levels in biological samples, such as fish (Tsutsumi et al. 2003) and breast milk (Leng et al. 2009), as well as in different environmental matrices, including sediments and soils (Brown et al. 2007, Dindal et al. 2007). As compared with HRGC/HRMS, the CALUX bioassay is a cost-effective high-throughput screening method, particularly for highly dioxin-contaminated samples from the environment and foodstuff (Hoogenboom et al. 2006). By stable transfection of Huh7 with a DRE-driven firefly luciferase reporter plasmid (4 × DRE-TATA-Luc),

a recombinant dioxin-responsive element (DRE)-driven luciferase expression cell line, Huh7-DRE-Luc, has been previously established (Chao et al. 2006, 2007, 2009). These studies revealed that some chemicals with no aryl hydrocarbon receptor (AhR) ligand activity—i.e., As^{3+}, arecoline, and Cd^{2+}—inhibited the TCDD-induced cytochrome P450 1A1 (CYP1A1) activation and also interfered with the TCDD-induced DRE-driven luciferase expression. The results suggest that the extensive cleanup procedure is critical for dioxin detection using the DRE-driven luciferase bioassay. In the present study, we combine an effective cleanup system with a DRE-driven luciferase bioassay for analysis of PCDD/F TEQ levels in the sediment and soil samples.

Materials and Methods

All solvents were pesticide residue grade from Merck (Darmstadt, Germany), Tedia (Fairfield, USA), or Sigma–Aldrich (St. Louis, USA). Standard solutions of PCDD/Fs (1613-LCS, labeled compounds stock solution; 1613-ISS, internal standard spiking solution; 1613-CSS, cleanup standard spiking solution; 1613CVS, EPA Method 1613 calibration and verification solution) were purchased from Wellington Laboratories (Ontario, Canada). Silica gel (100–200 mesh) was obtained from Fisher (Leicestershire, England). A rapid cleanup system by coupled acid silica column-activated carbon mini-column (CAPE-coupled carbon-acid silica column) was developed by CAPE Technologies (South Portland, USA).

The environmental samples of sediments (n = 10) and soils (n = 11) were obtained from dioxin-contaminated areas. Ten grams of solid samples were extracted with 300 ml toluene by Soxhlet extraction. Extracted samples were evaporated to near dryness and then transferred to the CAPE-coupled carbon-acid silica column for cleanup. The cleanup procedure using the CAPE- coupled carbon-acid silica column was previously described in detail (Chen et al. 2007, Lee et al. 2009). For the HRGC/HRMS method, all samples were added with the different PCDD/F internal standards before extraction and the labeled cleanup standards for PCDD/Fs analysis were added before the CAPE-coupled carbon-acid silica column cleanup. The extract was analyzed using a HRGC/HRMS (HP6890/JEOL JMS-700) equipped with a DB-5MS 60 m column. The quality of QA/QC met the criteria of the Taiwanese EPA Method.

For the DRE-driven luciferase bioassay, the labeled standards were not added in the extraction and cleanup procedure. Following the extraction and cleanup procedure, final extracts were evaporated to dryness and dissolved in dimethyl sulfoxide (DMSO) (100 µl) for the DRE-driven luciferase bioassay as described in detail in our previous report (Chao et al. 2006). In brief, Huh7-DRE-Luc cells were seeded at a density of 1×10^4 cells/well on a 96-well white plate (cat. 36101, Nalge Nunc, Denmark) with DMEM medium. Following incubation for 24 h, the cells were treated with the environmental extracts for 24 h. In parallel, the cells were also subjected to different concentrations of TCDD treatments (0.1, 0.3, 1, 3, 10, 30, 100, 300, 1,000, 3,000, and 10,000 pM) for 24 h for generation of a calibration curve of the DRE-driven luciferase activation by TCDD. After treatments, 50 µl of 1× lysis buffer (Promega, Madison, WI, USA) was added per well. To assure complete cell lysis, cells were freeze–thawed one time using liquid nitrogen and then were vortexed in 90 rpm at 37°C for 10 min. Luciferase activity was measured using Luciferase Assay System (Promega, Madison, WI, USA) according to the standard protocol provided. The luciferase activity was expressed as relative light units (RLU)/well. The sigmoid semi-logarithmic dose–response of TCDD calibration curve was fitted by the Hill equation (Chao et al. 2006).

WHO-TEQ levels of PCDD/Fs in samples were calculated based on the WHO_{2005} toxic equivalent factor (TEF) in the HRGC/HRMS method (chemical assay) (Van den Berg et al. 2006), meanwhile DL-TEQ levels in samples were extrapolated by the TCDD calibration curve from luciferase activity readings in the bioassay method. In this study DL-TEQs of each sample were independently measured three times in triplicate, and the differences in TEQ values (DL-TEQ vs. WHO-TEQ) between the DRE luciferase bioassay and the HRGC/HRMS method were examined using the paired T tests. Correlations of DL-TEQs and WHO-TEQs were performed by Spearman's rho correlation coefficient tests. DL-TEQs was predicted using the parameters of seventeen 2,3,7,8-substitued PCDD/F congeners, PCDDs, PCDFs, and PCDD/Fs by testing multiple linear stepwise regression models. For estimation of PCDD/F WHO-TEQs, DL-TEQs were also fitted by a proper equation. The statistics in this study were performed with SPSS 12.0 version.

Results and Discussion

As seen in Table 1, means of DL-TEQs range from 65.9 to 2,670 ng-DL-TEQ/kg dry weight (d.w.) for 10 sediment samples and from 74.3 to 891 ng-DL-TEQ/kg d.w. for 11 soil samples. The values of relative standard deviation (RSD) are between 3.8 and 20.0% in these 21 samples. Although DRE-driven luciferase bioassay (or DRE-CALUX) is a fast-screen method, unknown contaminants in extracts may easily interfere with measurements of bioassay. In our previous reports using Huh7-DRE-Luc cells (Chao et al. 2006, 2007, 2009), both arsenite (As^{3+}) and

Table 1 Measurements of PCDD/F levels in 10 sediment and 11 soil samples from the contaminated area using DRE-driven luciferase bioassay

Sample	Mean (n = 3) (ng-DL-TEQ/kg d.w.[a])	Range (ng-DL-TEQ/kg d.w.)	RSD[b] (%)
Sediment 1	213	185–239	12.9
Sediment 2	123	120–126	2.52
Sediment 3	126	97.6–143	20.0
Sediment 4	65.9	63.1–68.0	3.80
Sediment 5	518	473–578	10.5
Sediment 6	1,660	1,490–1,960	15.6
Sediment 7	113	103–124	9.29
Sediment 8	78.4	65.8–92.0	16.7
Sediment 9	2,670	2,400–3,160	15.7
Sediment 10	1,450	1,340–1,570	7.86
Soil 1	582	528–641	9.72
Soil 2	891	869–926	3.42
Soil 3	599	551–683	12.3
Soil 4	154	144–162	5.88
Soil 5	59.0	52.9–66.4	11.6
Soil 6	75.1	66.6–83.8	11.4
Soil 7	74.3	65.9–79.5	9.87
Soil 8	89.6	72.2–107	19.4
Soil 9	182	158–195	11.5
Soil 10	84.7	74.2–97.1	13.6
Soil 11	102	90.3–109	9.64

[a] ng-DRE-driven luciferase-TEQ/kg d.w

[b] Relative standard deviation (coefficient variation)

cadmium (II) (Cd^{2+}) inhibited the DL-TEQ activation, whereas arecoline enhanced the DL-TEQ activation. Hoogenboom et al. (2006) demonstrated that extensive cleanup procedures would increase precision of DRE-CALUX bioassay due to the following two reasons. First, absence of spiked internal standards in the samples did not estimate the loss in cleanup. Second, the contaminants, particularly for unknown AhR agonists and antagonists, in solvents and samples might easily interfere with luciferase activation. Therefore, both extensive cleanup procedure and a highly sensitive detection system are critical for the precision measurements using DRE-driven luciferase bioassay. It has been previously shown that analysis of dioxins using a CAPE-coupled carbon-acid silica column together with HRGC/HRMS meets the QA/QC criteria of Taiwan EPA (Chen et al. 2007, Lee et al. 2009). We also showed that our DRE-driven luciferase bioassay, with a semi-logarithmic dose–response calibration curve (a sigmoid appearance with $R^2 = 0.995$, $p < 0.001$), was used in PCDD/Fs analysis (Chao et al. 2006).

Correlations between DRE-driven luciferase bioassay and HRGC/HRMS method are shown in Fig. 1. Levels of DL-TEQs and WHO-TEQs in 21 samples are logarithmically transformed to an approximately normal distribution. We find that log DL-TEQs is positively correlated with log PCDD/Fs WHO-TEQs ($R^2 = 0.811$, $p < 0.001$), log PCDDs WHO-TEQs ($R^2 = 0.813$, $p < 0.001$), log PCDFs WHO-TEQs ($R^2 = 0.805$, $p < 0.001$), and log TCDD WHO-TEQs ($R^2 = 0.910$, $p < 0.001$), respectively. In addition, DL-TEQ levels in our samples were not linearly associated with WHO-TEQ levels prior to logarithm transformation. Several studies of samples from soils and sediments have also revealed nonlinear correlations between DL-TEQs and WHO-TEQs (Brown et al. 2007, Dindal et al. 2007, Kanematsu et al., 2009). Following the paired T tests of DL-TEQs and WHO-TEQs, a mean of the paired differences (DRE-driven luciferase-HRGC/HRMS) is 183 ng-TEQ/kg d.w. ($p < 0.001$). The DL-TEQ level is significantly higher than the PCDD/F WHO-TEQ level in each sample. False-positive findings are always observed in the present study. It has been reported that DL-TEQ levels were also consistently higher than PCDD/F WHO-TEQ levels in soils and sediments (Brown et al. 2007, Dindal et al. 2007).

In Table 2, log DL-TEQ levels are significantly predicted by log values of 2,3,7,8-TCDD (B = 72.2, R^2 change = 0,941, $p < 0.001$) and 1,2,3,7,8-PeCDD (B = 9.71, R^2 change = 0,022, $p < 0.001$) by multiple stepwise linear regression models (adjusted $R^2 = 0.962$, $p < 0.001$). Our results show that 2,3,7,8-TCDD levels are the most important predictor among 17 substituted 2,3,7,8-PCDD/Fs for estimating DL-TEQ levels. For consideration of cost and application in the future dioxins survey, PCDD/F WHO-TEQ levels must be effectively predicted by DL-TEQ levels. To describe the relationship between DL-TEQs and WHO-TEQs of 21 samples from soils or sediments in this study, a mathematical model was also developed: log (PCDD/Fs WHO-TEQ) = 0.992 × (log DL-TEQs)–0.00014 × (log DL-TEQs)^2–81.4, with R^2 = 0.914. Because the equation exhibits a highly correlated relationship between DL-TEQs and WHO-TEQs, the CAPE-coupled carbon-acid silica column together with DRE-driven luciferase bioassay may be a powerful tool to predict PCDD/Fs WHO-TEQ levels in samples from soils or sediments. Nonlinear mathematical models used to describe the significantly high correlations between WHO-TEQs and DL-TEQs (or DRE-CALUX-TEQs) in samples from soils or sediments were performed after logarithm transformation of TEQs (Brown et al. 2007, Dindal et al. 2007, Kanematsu et al. 2009).

Kanematsu et al. (2009b) demonstrated that combined use of DRE-driven luciferase bioassay (or DRE-CALUX) and HRGC/HRMS provided new insights into transport and fate of PCDD/Fs in the environment. This study further reveals that the CAPE-coupled carbon-acid silica column

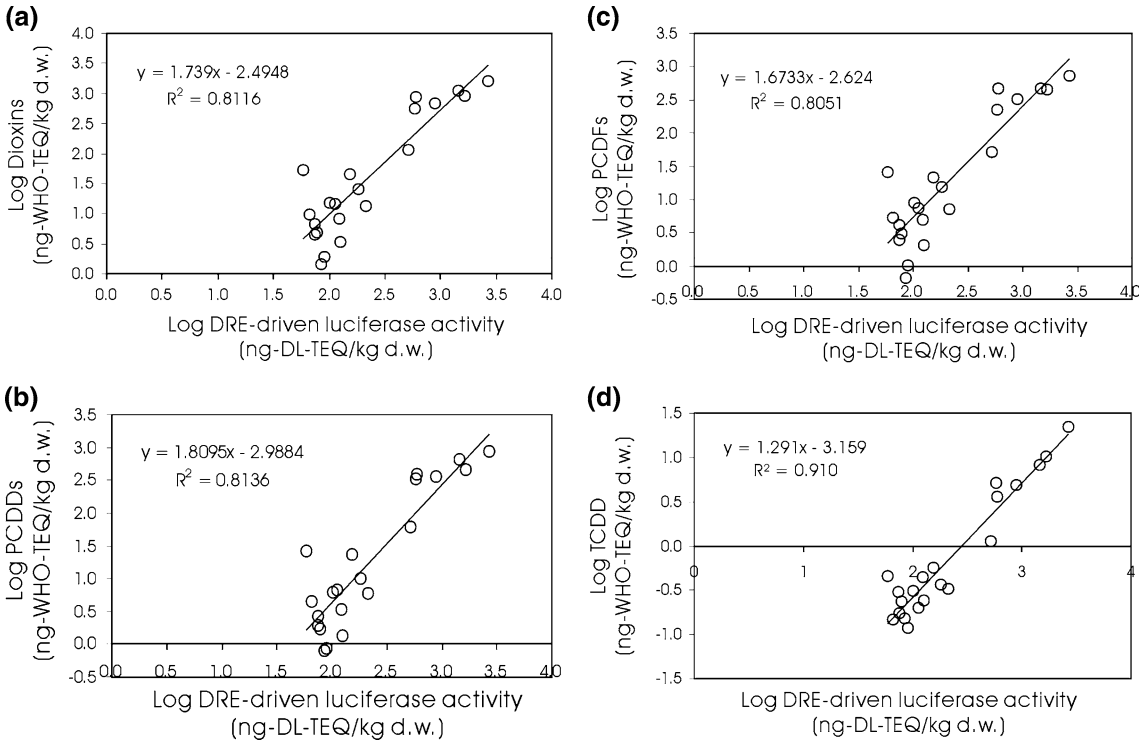

Fig. 1 Correlations between bioassay (DRE-driven Luciferase bioassay) and chemical assay (HRGC/HRMS) are demonstrated by **a** DL-TEQs (bioassay) versus PCDD/Fs WHO-TEQs (chemical assay), **b** DL-TEQs versus PCDDs WHO-TEQs, **c** DL-TEQs versus PCDFs WHO-TEQs, and **d** DL-TEQs vs. 2,3,7,8-TCDD WHO-TEQs

Table 2 Multivariate analysis of DL-TEQs using 17 2,3,7,8-substituted PCDD/F congener TEQs, PCDD TEQs, PCDF TEQs, and PCDD/F TEQs as the predictors by testing the multiple linear stepwise regression model

Dependent[a]	Independent[b]	B	Adjusted R^2	R^2 change	F change	*p*
DRE-driven luciferase	2,3,7,8-TCDD	72.2	0.962	0.941	974	<0.001***
	1,2,3,7,8-PeCDD	9.71		0.022	35.7	<0.001***

[a] Log DL-TEQs (ng-DL-TEQ/kg d.w.)

[b] Log WHO-TEQs (ng-WHO_{2005}-TEQ/kg d.w.)

*** $p < 0.001$

together with DRE-driven luciferase bioassay is a powerful green technique for the fast-screening of dioxins. Thus, combination of this green technique and HRGC/HRMS may significantly facilitate large-scale dioxins surveys, particularly for heavily contaminated areas and the outbreak of dioxin contamination, in a more efficient and cost-effective way. In the present study, PCDD/Fs WHO-TEQs are effectively predicted by DL-TEQs using our mathematical model in the soil and sediment samples. The analytical method used in the present study has been shown to be a valuable tool for high-throughput screening of PCDD/F TEQ levels in environmental samples, particularly from soils and sediments in hotspot areas.

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