Evaluation of mixture effects in a crude extract of compost using the CALUX bioassay and HPLC fractionation

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Introduction

The toxic equivalency factor (TEF) approach has been used to assess the risk associated with mixtures of halogenated aromatic hydrocarbons (HAHs), such as PCDDs, PCDFs, and coplanar PCBs. The TEF of a compound represents its toxicity relative to that of 2,3,7,8-TCDD, and TEF values for individual compounds are used to determine the toxic equivalent quantity (TEQ) for mixtures of PCDDs, PCDFs, and coplanar PCBs[1]. The TEF approach can only be applied to arylhydrocarbon receptor (AHR)-mediated responses and does not take into account the modulating effects of compounds that do not show AHR binding activity. AHR-based bioassays have been used to determine induction equivalency factors (IEFs) for CYP1A-inducing PAHs, and the IEFs have in turn been used to calculate induction equivalent quantities (IEQs)[2,3]. Both the TEF approach and the IEF approach are based on an additivity model, and they can be applied only when the toxicity of PACs adheres to this model.

In general, crude extracts and polyaromatic hydrocarbon (PAC)-containing fractions of environmental samples show high activities in the AHR-based bioassay. Although many studies have tried to determine which compounds contribute to this activity based on additivity theory, these values are higher than the corresponding chemically calculated IEQs or TEQ values[4,5]. These compounds in environmental samples exist as mixtures, and interactions between compounds must be considered in chemical risk assessments. In particular, if synergistic interactions occur in environmental samples, application of the additivity assessment would underestimate real chemical risk.

In this study, we investigated mixture effects in an extract of an environmental sample. First we established a simple fractionation procedure for the sample, and then we examined the retention characteristics of PACs using RP-HPLC on an octadecylsilica (ODS) column. Then we applied HPLC fractionation and combined CALUX (DR-CALUX®: Dioxin-Responsive Chemical-Activated Luciferase gene eXpression)/chemical analysis to a night soil sludge (NSS) compost extract to determine which compounds contributed to the CALUX-TEQ (2,3,7,8-TCDD equivalent) of the crude extract. Finally, to assess the mixture effects, we exposed the HPLC fractions to CALUX cells in the presence of 2,3,7,8-TCDD at concentration levels similar to those in the

original compost sample and determined whether there was a synergistic interaction between the sample fraction and 2,3,7,8-TCDD in terms of CALUX activity.

Materials and Methods

***Compost sampling and sample preparation*:** The sampling procedures have been described elsewhere[4]. A NSS compost sample was obtained from a night soil treatment plant in Nagasaki, Japan. The compost material was prepared from dewatered sludge, which was inoculated and introduced to a rotary drum with a retention time of 14 days. Compost was produced at the rate of 1.0 t/day. Approximately 150g of air-dried and ground samples (particle size < 2 mm) were subjected to alkali (1 M potassium hydroxide/ethanol) digestion for two hours. After the addition of water, the mixture was filtered through a glass fiber, and the filtrate was extracted three times with *n*-hexane. The *n*-hexane fraction was washed with 2% sodium chloride/water and dehydrated. A portion of the fraction (equal to 10 g of dry sample) was extracted with DMSO. The DMSO fraction was diluted with water and followed by the re-extraction with *n*-hexane. The extract was dehydrated and then evaporated almost to dryness under a nitrogen flow. The residue was redissolved in 70 µl of DMSO as crude extract and stored at –20 °C for subsequent CALUX assay and HPLC fractionation.

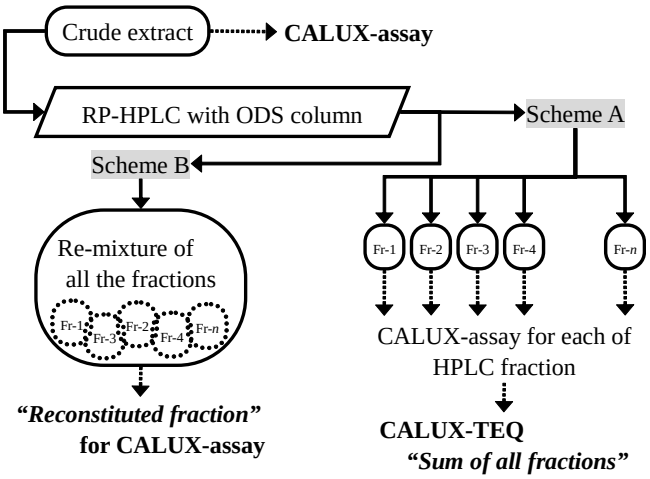


Fig. 1. The fractionation scheme for the crude extract of night soil sludge compost.

***RP-HPLC fractionation*:** 26 PACs and samples were separated on an ODS column (Wakosil-PAHs, 4.6 × 250 mm, 5 µm, WAKO, Japan) and a guard column (4.6 × 50 mm) at 30 °C. The extract was eluted with the following solvent system at a flow rate of 1 ml/min: 0–4.0 min, 10% acetonitrile in methanol/water (80/20, V/V); 4.0–7.0 min, a linear gradient of 10 to 100% acetonitrile in methanol/water (80/20, V/V); 7.0–50.0 min, 100% acetonitrile. Standard compounds were identified using a photodiode array detector (Agilent, USA). The peaks detected in the range from 210 to 360 nm in the UV absorption spectrum were used to fix the retention times. For HAHs, the peaks detected by CALUX assay were used to fix the retention times. An overview of the fractionation scheme for the NSS compost is shown in Fig. 1. A 10-µl sample was injected and then fractionated over a period of 50.0 min (Fig. 1, Scheme A): fractions were collected at 30-s intervals from 0 to 25.0 min and at 60-s intervals from 25.0 to 50.0 min. Furthermore, in order to determine the amount of sample lost through fractionation, all the fractions were recombined to form a reconstituted fraction (Fig. 1, Scheme B). All the fractions were evaporated, and the residue was taken up in 30 µl of DMSO and then assessed for dioxin-like activity using the CALUX assay.

***Evaluation of mixture effects by co-exposure of the fractionated samples and 2,3,7,8-TCDD to the CALUX cells*:** On the basis of the additive theory, we co-exposed the CALUX cells to the fractionated samples and 2,3,7,8-TCDD. The amounts of added 2,3,7,8-TCDD were decided on the basis of the TEQ concentrations actually contained in the NSS compost sample. The values in the NSS compost were 5.1 pg WHO-TEQ/g for PCDDs, PCDFs, and coplanar PCBs and 29 pg CALUX-TEQ/g for a HAH fraction after cleanup[3]. Referring to these values, we decided on 2,3,7,8-TCDD concentrations of 0.024 pg/µl, 0.081 pg/µl, 0.24 pg/µl, and 0.81 pg/µl, which correspond to 0.96 pg, 3.2 pg, 9.6 pg, and 32 pg CALUX-TEQ/g. After mixing the same quantity of 2,3,7,8-TCDD into a sample fraction, we performed the CALUX assay to evaluate mixture effects.

***DR-CALUX assay*:** The rat hepatoma H4IIE cell line, stably transfected with an AHR-regulated luciferase gene construct, was obtained from Bio Detection Systems B.V. (Amsterdam, Netherlands). The CALUX assay was performed as previously described by Takigami et al.[4]. The 2,3,7,8-TCDD calibration curve was fitted by using a following curve fit (Slide Write Plus Version 6.00 software, Advanced Graphics Software, USA): $y = a_0/[1 + (x/a_1)^{a2}]$. After correcting for background activity (DMSO control), the luciferase activities of sample extracts that exhibited responses between the limit of quantification and 5 pM 2,3,7,8-TCDD were interpolated onto the fitted 2,3,7,8-TCDD calibration curve in order to calculate CALUX-TEQs per gram of sample.

Results and Discussion

***Crude activity in terms of bio/chemical TEQs and activity pattern using the CALUX assay and RP-HPLC fractionation*:** The concentrations of PCDD/Fs, coplanar PCBs, and PAH_{16} and the CALUX-TEQs for the NSS compost are shown in Table 1. The crude extract of the NSS compost had significant CALUX activity, and its activity was higher than that of an acid-resistant extract from which labile compounds such as PAHs had been removed by reflux treatment with 44% sulfuric acid silicagel[4]. The activity of the crude extract was 360 pg CALUX-TEQ/g, which was approximately 30 times that of the acid-resistant fraction and 70 times the WHO-TEQ value chemically calculated from WHO-TEFs[1] (Table 1). Although we considers that the contribution of PAH_{16} explains the higher CALUX-TEQ, the theoretical CALUX-TEQ for PAH_{16} (PAH_{16}-derived CALUX-TEQs) calculated chemically[4] is only approximately 10% of the CALUX-TEQ of the crude extract (Table 1). These results suggest that acid-labile dioxin-like compounds other than the 16 PAHs contribute strongly to the CALUX activity in the crude extract.

Table 1. Results of chemical analysis and CALUX of the compost sample. [a] Data cited from Takigami et al.[4].

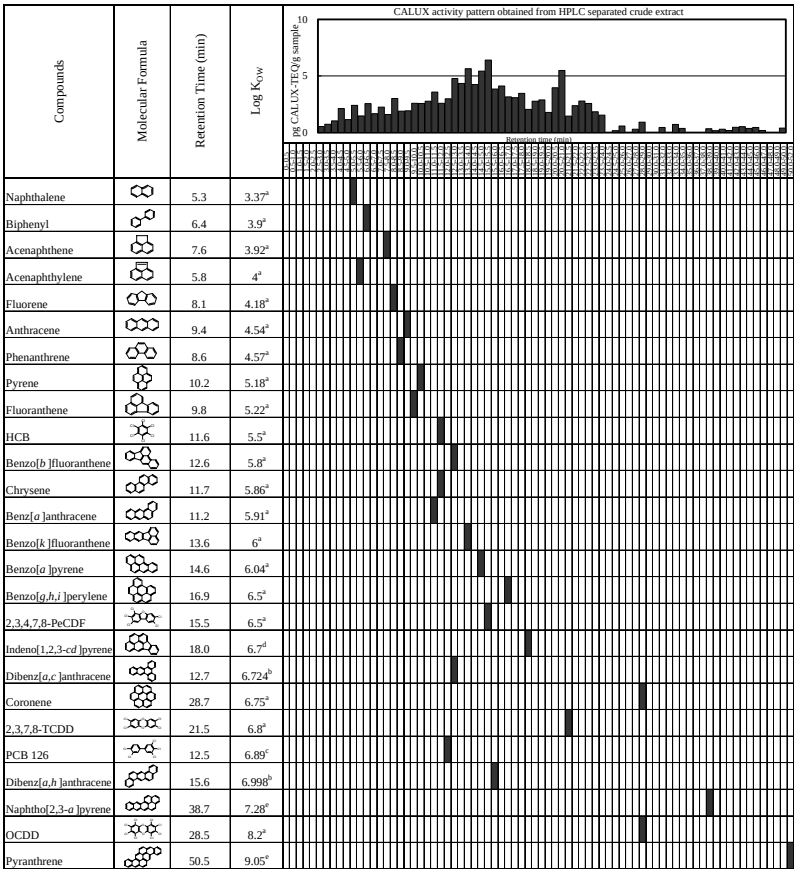
Night soil treatment sludge compost[pg/g-dry]					
PCDD/Fs [a]	260	*16 PAHs specified by U.S. EPA* [a]	210000	***CALUX-TEQ***	
Coplanar PCBs [a]	4400	*2-3 ring PAHs* $_{16}$	35000	***Crude extract***	360
		4-6 ring PAHs $_{16}$	170000	***Acid-resistant extract*** [a]	11
PCDD/F WHO-TEQ [a]	3				
Coplanar PCB WHO-TEQ [a]	2.1	***PAH*** $_{16}$ ***-derived CALUX-TEQ*** [a]	34		
Total WHO-TEQ [a]	5.1				

The CALUX activity patterns of the crude separated fractions were investigated by means of the CALUX assay and RP-HPLC (Fig. 1: Scheme A). To determine the active compounds that contributed to the fractionated CALUX activity, we investigated the elution characteristics of 26 HAHs and PAHs by RP-HPLC on an ODS column. The CALUX activity pattern of the crude extract and the molecular structures, retention times, and log *n*-octanol/water partition coefficients

(log K_{OW}) values[6-8] for the tested standards are shown in Fig. 2. For indeno[1,2,3-*cd*]pyrene, the log K_{OW} value was obtained from the Hazardous Substances Data Bank (HSDB) via TOXNET (http://toxnet.nlm.nih.gov/). Since the log K_{OW} values for naphtho[2,3-*a*]pyrene and pyranthrene could not be found anywhere, we calculated them using the Log K_{OW} Program (http://esc.syrres.com/interkow/LogKow.htm), which predicts the log K_{OW} values of organic compounds using an atom/fragment contribution method[9]. The standards tended to elute according to their log K_{OW}. The fraction taken at 15.0 to 15.5 min showed the highest activity. The fractions taken at 13.5 to 14.0 min and at 20.5 to 21.0 min also showed relatively high activities, and CALUX activity was barely detectable in the fractions taken after 24.0 min. Taking the elution results for the standards into consideration, the CALUX activity pattern in the crude extract of the NSS compost suggested that the contribution of dioxin-like compounds with log K_{OW} values of 6.0 to 7.0 was high, and the compounds with log K_{OW} values less than 6.0 also showed relatively high activity (Fig. 2).

***Quantitative comparison of the CALUX activity in the crude extract and its constituent HPLC fractions*:** To determine whether the activities of the dioxin-like compounds in the NSS compost could be assessed by the additivity theory, we compared the CALUX activity of the crude extract with the arithmetical sum of the CALUX activities of all the fractions separated by RP-HPLC. The CALUX-TEQ of the crude extract was three times the CALUX-TEQ sum of all the fractions (Fig. 3). Although Brack et al.[10] confirmed typical recoveries of 60 to 80% for chemical analysis, they stated that a difference between the EROD-IEQs of a crude extract and its reconstituted fraction mixture was caused by matrix effects in the complex mixture, evaporation, or incomplete dissolution during solvent exchange[5]. For above reasons about recovery loss, to evaluate the recovery of CALUX activity through RP-HPLC fractionation, we re-collected all fractions after RP-HPLC separation and prepared a reconstituted fraction according to the method of Brack and Schirmer[5] (Fig. 1: Scheme B). The activity of the reconstituted fraction was 260 pg CALUX-TEQ/g, so the overall recovery rate for the CALUX-TEQ through RP-HPLC fractionation was 72%, which agrees with the recovery rates Brack et al.[10] reported.

The CALUX activity of the reconstituted fraction was twice the arithmetical sum of the CALUX activity of all the separated sediment fractions (in this case, recovery loss need not be considered) (Fig. 3). Houtman et al.[11] reported that the CALUX activity of an untreated extract was only 50% of the sum of the activity found in gel permeation chromatography fractions, and proposed that this "non-additive" reduction may be caused by unknown AHR antagonists. In contrast, our results confirmed the "non-additive" increase of CALUX activity under the same evaluation method in the investigated crude compost extract. The additive theory has been incorporated into the TEF approach, which has been used to assess the risks associated with mixtures of HAHs such as dioxins[1]. Moreover, this theory has been also applied to PAH mixtures for hazard assessment[2,3] because the toxic characteristics of some PAHs are similar to those of HAHs[12]. However, Basu et al.[2] have used test experiments with reference compounds to determine that this additive assessment is not applicable to mixtures containing PACs with different potency and efficacy in the EROD bioassay. Therefore, to determine whether a "non-additive" increase occurred in the crude extract of the NSS compost, we co-exposed the fractionated samples and 2,3,7,8-TCDD to the CALUX cells.



Compounds	Molecular Formula	Retention Time (min)	Log K_{OW}
Naphthalene		5.3	3.37[a]
Biphenyl		6.4	3.9[a]
Acenaphthene		7.6	3.92[a]
Acenaphthylene		5.8	4[a]
Fluorene		8.1	4.18[a]
Anthracene		9.4	4.54[a]
Phenanthrene		8.6	4.57[a]
Pyrene		10.2	5.18[a]
Fluoranthene		9.8	5.22[a]
HCB		11.6	5.5[a]
Benzo[*b*]fluoranthene		12.6	5.8[a]
Chrysene		11.7	5.86[a]
Benz[*a*]anthracene		11.2	5.91[a]
Benzo[*k*]fluoranthene		13.6	6[a]
Benzo[*a*]pyrene		14.6	6.04[a]
Benzo[*g,h,i*]perylene		16.9	6.5[a]
2,3,4,7,8-PeCDF		15.5	6.5[a]
Indeno[1,2,3-*cd*]pyrene		18.0	6.7[d]
Dibenz[*a,c*]anthracene		12.7	6.724[b]
Coronene		28.7	6.75[a]
2,3,7,8-TCDD		21.5	6.8[a]
PCB 126		12.5	6.89[c]
Dibenz[*a,h*]anthracene		15.6	6.998[b]
Naphtho[2,3-*a*]pyrene		38.7	7.28[e]
OCDD		28.5	8.2[a]
Pyranthrene		50.5	9.05[e]

Fig. 2. The CALUX activity pattern obtained from a crude extract of night soil sludge compost and the molecular structures, retention times, and log K_{OW} values of 26 standards tested by RP-HPLC using an ODS column. [a, b and c] Data cited from Mackay et al.[6], Ferreira[7] and Hawker and Connell[8]. [d] Quoted from the Hazardous Substances Data Bank (HSDB) via TOXNET (http://toxnet.nlm.nih.gov/). [e] Calculated using the Log K_{OW} Program (http://esc.syrres.com/interkow/Log Kow.htm).

***Evaluation of mixture effects in the crude extract by co-exposure of fractionated samples and 2,3,7,8-TCDD to the CALUX cells*:** We added 0.024, 0.081, 0.24, and 0.81 pg/µl of 2,3,7,8-TCDD (corresponding to 0.96, 3.2, 9.6, and 32 pg CALUX-TEQ/g) into each sample fraction. The added 2,3,7,8-TCDD was based on the actual TEQ concentration in the NSS compost. If compounds interact additively, the activity of the mixtures to which 2,3,7,8-TCDD is added should be the sum of the 2,3,7,8-TCDD-derived CALUX-TEQ and the sample-derived CALUX-TEQs. The ratios, which were calcurated by the predicted CALUX activity patterns of the sample at the four 2,3,7,8-TCDD levels and the experimental CALUX activity patterns, clearly indicated that the experimental CALUX activities from 24.0 to 50.0 min tended to be higher than the predicted activities, although the ratio depended on the 2,3,7,8-TCDD level (Fig. 4). At the lowest 2,3,7,8-TCDD level, the ratios from 24.0 to 33.0 min were two to three times as high as predicted CALUX activities, while the ratios from 2.5 to 24.0 min were less than 1. At the highest 2,3,7,8-TCDD level, the ratio of experimental and predicted CALUX activities decreased in the elution range between 24.0 and 50.0 min. However, at all the 2,3,7,8-TCDD levels, the ratio tended to increase in the elution range between 24.0 and 50.0 min. Although these results suggest that the strength of the mixture effect changes with the ratio of compounds to 2,3,7,8-TCDD, "non-additive" increases were consistently observed for the elution range between 24.0 and 50.0 min.

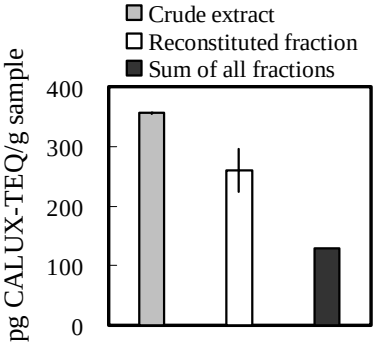


Fig. 3. The CALUX-TEQ of a crude extract from night soil sludge compost (crude extract) and the reconstituted mixture of separated crude fractions (reconstituted fraction), and the sum of the CALUX-TEQs of the separated fractions (sum of all fractions). Values for the crude extract and the reconstituted fraction represent the mean ± S.D. from three independent assays.

We compared the retention times of the compounds that eluted in the "non-additive" retention range with the retention times of the standards tested by RP-HPLC with the ODS column.

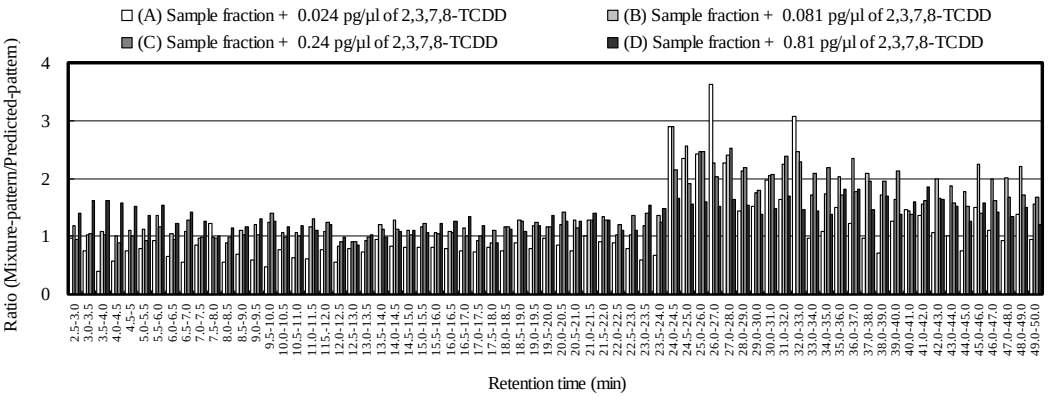


Fig. 4. The ratio of experimental and predicted CALUX activities for each fraction taken over the retention time range from 2.5 min to 50.0 min. The amounts of 2,3,7,8-TCDD added to the sample fractions were (A) 0.024, (B) 0.081, (C) 0.24, and (D) 0.81 pg/µl (corresponding to 0.96, 3.2, 9.6, and 32 pg CALUX-TEQ/g).

Compounds that elute in the range from 24.0 to 50.0 min are likely to be not less than log K_{OW} value of 6.0 (Fig. 2). Compounds such as PAHs with six or more aromatic rings and some PCDDs, PCDFs, and coplanar PCBs may correspond to the compounds that eluted from 24.0 to 50.0 min. Alkyl PAHs, which were not investigated in this study, are reported to elute later than their parent PAHs[10], and alkyl derivatives of low-ring PAHs may elute in the range where the remarkable synergistic rise in the experimental/predicted CALUX-TEQ ratios was observed. The results from co-exposure of the sample fraction and 2,3,7,8-TCDD suggest that, at least in this study, hydrophobic compounds which are log K_{OW} value of 6.0 or more exhibit a "non-additive" increase in CALUX activity in the presence of 2,3,7,8-TCDD. Synergistic interactions, that is, non-additive interactions, have been reported for PAHs[2] and HAHs[13]. In order to identify these compounds, it is indispensable to utilize not only hydrophobicity but also other physicochemical properties for a fractionation. It might be possible to understand the physicochemical properties of compounds that exhibit synergistic increases in CALUX activity in crude extracts of environmental samples and to identify/quantify these compounds using a useful bio/chemical analysis method.

In conclusion, our results demonstrate that compounds in NSS compost may interact synergistically with 2,3,7,8-TCDD in dioxin-like activity. This finding points out the necessity for detailed investigation of synergistic effects in environmental samples.

Acknowledgements

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