



Considerations for potency equivalent calculations in the Ah receptor-based CALUX bioassay: Normalization of superinduction results for improved sample potency estimation

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ABSTRACT

The chemically activated luciferase expression (CALUX) system is a mechanistically based recombinant luciferase reporter gene cell bioassay used in combination with chemical extraction and clean-up methods for the detection and relative quantitation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related dioxin-like halogenated aromatic hydrocarbons in a wide variety of sample matrices. While sample extracts containing complex mixtures of chemicals can produce a variety of distinct concentration-dependent luciferase induction responses in CALUX cells, these effects are produced through a common mechanism of action (i.e. the Ah receptor (AhR)) allowing normalization of results and sample potency determination. Here we describe the diversity in CALUX response to PCDD/Fs from sediment and soil extracts and not only report the occurrence of superinduction of the CALUX bioassay, but we describe a mechanistically based approach for normalization of superinduction data that results in a more accurate estimation of the relative potency of such sample extracts.

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1. Introduction

Halogenated aromatic hydrocarbons (HAHs), such as the polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs), are an environmentally and metabolically persistent class of highly toxic chemicals. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent HAH, and related “dioxin-like” HAHs (dl-HAHs) can produce a wide variety of species and tissue-specific toxic and biological effects, including immuno- and hepato-toxicity, endocrine disruption, cancer and lethality [1–5]. It has been well established that the majority of the toxic/biological effects of dl-HAHs are mediated by the Ah receptor (AhR), a soluble intracellular ligand-dependent transcription factor [1,2,5–8], and that AhR-dependent toxicity requires persistent activation of the AhR signaling pathway by metabolically stable dl-HAHs [1,2,5,9].

When assessing the toxicity and analysis of HAHs present in environmental and biological samples, one must take into consideration that there exists a total of 209 different PCB, 135 different PCDF and 75 different PCDD congeners, of which TCDD-like biological/toxicological effects are produced by a relatively

small number of these congeners [2,10]. Toxic equivalency factors (TEFs) for those individual HAH congeners producing *in vivo* “dioxin-like” toxicity have been established by the World Health Organization and these values represent the toxic potency of the specific dl-HAH congener expressed relative to that of the most toxic congener, TCDD [10]. Determination of the toxic potency of dl-HAHs present in a given sample extract first requires the application of sophisticated cleanup procedures followed by high-resolution instrumental analysis (capillary gas chromatography and high resolution mass spectrometry (GC/HRMS)) for the separation, identification, and quantitation of individual PCDD, PCDF and PCB congeners [11–14]. Calculation of the relative toxic equivalency (TEQ) or toxic potency of a complex mixture of dl-HAHs has been extensively described and reviewed [2–10,14]. Although the instrumental analysis methodology approach is considered the “gold standard” for measurement of HAHs in sample extracts and ultimately for determination of sample TEQs, these analyses require highly sophisticated equipment and training and can be very costly and time-consuming. Accordingly, numerous rapid and relatively inexpensive *in vitro* and cell-based bioanalytical approaches have been developed that are capable of detecting and estimating the relative potency of complex mixtures of dl-HAHs, many of which take advantage of the AhR-dependent signal transduction mechanism of action [13–20].

The chemically activated luciferase expression (CALUX) recombinant cell bioassay is one such bioassay system that has been extensively utilized for the detection and relative quantitation of

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dl-HAHs in sample extracts, although numerous other cell bioassays have been described (reviewed in [13,14,17–19,21]). The CALUX bioassay system takes advantage of the AhR-dependent signal transduction mechanism of action of TCDD and dl-HAHs and utilizes recombinant mammalian cell lines that contain a stably transfected AhR-responsive firefly luciferase reporter gene that responds to dl-HAHs and other known AhR agonists with the induction of luciferase in a dose-, time-, chemical- and AhR-dependent manner [13,18,19,21]. Although the highest affinity and most potent activators of the AhR signaling pathway are TCDD and related dl-HAHs, recent evidence has shown that the AhR can be activated by an extremely diverse range of chemical ligands whose structure and physiochemical characteristics are dramatically different than that of dl-HAHs [8,20,22–24]. Accordingly, accurate assessment of the relative potency of dl-HAHs present in a complex chemical mixture requires removal of undesired AhR active substances by chemical fractionation and clean-up procedures [13,14,19,25–27]. The CALUX bioassay when coupled with an appropriate sample extract clean-up method has been used successfully for accurate detection and relative quantitation of dl-HAHs in a wide variety of biological, environmental and food/feed samples (reviewed in [13,19]) and has received regulatory certification as a validated method by the US EPA (Method 4435) for determination of dl-HAHs in various matrices [28]. Numerous studies have reported that potency estimates for a given sample determined by CALUX and GC/HRMS typically exhibit a reasonably high degree of correlation [13,19,26,27]. However, these same studies also revealed that potency estimates determined by CALUX and other cell bioassays commonly yield values (referred to here as bioanalytical equivalents (BEQs)) significantly higher than the TEQs calculated from GC/HRMS analysis of the same samples, particularly when environmental samples are analyzed. This overestimation by the bioassays has been suggested to result from differences between the TEF values for the dl-HAHs used for GC/HRMS TEQ calculations and their CALUX-based relative potency (REP) values [29] and/or the presence of other AhR active dl-HAHs (i.e. polybrominated and/or mixed chloro/bromo-dl-HAHs or other chemicals [19,30–32]) in sample extracts that are not measured using GC/HRMS methods established for chlorinated dl-HAHs. The recent demonstration that activation of protein kinase C and inhibition of protein synthesis can synergistically enhance AhR-dependent gene expression [33–37] suggests additional avenues by which chemicals in a complex mixture can enhance the output response of the CALUX bioassay leading to an inaccurate overestimation of sample potency (i.e. BEQ). While synergistic enhancement of AhR-dependent gene expression can be produced using purified compounds and these mechanisms in controlled experimental conditions, their relevance and significance for CALUX bioassay analysis of actual environmental samples remains to be confirmed. Here we report for the first time superinduction of the CALUX bioassay by the PCDD/Fs fraction extracted from soil/sediment samples and describe a mechanistically based approach for normalization of superinduction data that results in a more accurate estimation of the relative potency (BEQ) of such sample extracts.

2. Methods and materials

2.1. Chemicals and materials

TCDD was a kind gift from Dr. Stephen Safe (Texas A&M University). Luciferase substrate (luciferin) and lysis buffer were purchased from Promega, alpha minimum essential media (α MEM) from Gibco/Invitrogen and fetal bovine serum (FBS) from Atlanta Biologicals. White Costar 96 well clear-bottomed tissue culture

plates, pesticide residue grade solvents, silica gel, celite and granular sodium sulfate were purchased from Fisher Scientific. Sodium sulfate was baked at 450 °C for 4 h in an aluminum tray prior to use. All experimental glassware was borosilicate and was prepared by hand washing with a final triple rinse of Milli-Q water, air drying, and openings and ground glass contact surfaces covered with heavy duty aluminum foil and baked in a muffle furnace for a minimum of 4 h at 450 °C.

2.2. Sediment/soil sample extract preparation

Sediment and soil samples from a previous study [38] were obtained in a crushed, dry state ready for extraction. Approximately 2 g of sample was extracted with 10 ml of 80:20 toluene:methanol, followed by two extractions with 10 ml of toluene each. Individual extracts were allowed to settle for 20–30 min followed by decanting of the supernatant onto a layered filter column consisting of (from the bottom of the column up): glass wool fiber, baked sodium sulfate, celite and baked sodium sulfate (for removal of particulate material). The column was rinsed with an additional 10 ml of toluene and all flow-through eluates were combined and the solvent volume reduced to near dryness by vacuum centrifugation. Sample extracts were rinsed from the centrifuge tube and resuspended with 2 ml of hexane followed by four more 1 ml hexane rinses. Each rinse was transferred onto a 33% acid silica column where all rinses were composited, allowing for the separation of AhR active polycyclic aromatic hydrocarbons [27,28] from HAHs. The acid silica column was directly eluted onto an XCarb column (celite/1% XCarb column sandwiched between sodium sulfate and retained between glass wool plugs [27,28]), followed by three 5 ml hexane rinses of the acid silica column. This column combination allows for the separation of dl-HAHs into PCDD/F and PCB fractions [28]. The XCarb column was then separately rinsed with an additional 5 ml hexane solvent aliquot. Two separate fractions were eluted from the XCarb column: first PCBs and PCB-like compounds were eluted from the XCarb column with 15 ml of a 80/10/10 hexane/toluene/ethyl acetate solution, the XCarb column was then inverted and PCDD/Fs eluted from the XCarb column with 15 ml of toluene. All solvents were reduced to near dryness by vacuum centrifuge. Samples were rinsed from the centrifuge tube with 1 ml of hexane and this was repeated 3 more times (a total of 4 ml), each 1 ml rinse transferred to an amber, Teflon-capped 4 ml vial, sealed with parafilm, and stored wrapped in foil in the dark until analyzed. Only the PCDD/F-containing fraction was used for the CALUX bioassay studies described here. The concentration of PCDDs and PCDFs in these samples had been previously determined using standard GC/HRMS analysis [38] and TEQ values for each sample calculated using the 2005 TEF values [10].

2.3. Cell culture, chemical treatment and CALUX analysis

Recombinant mouse hepatoma (H1L6.1c3) CALUX cells were grown and maintained as we have previously described [18,21,28]. These cells contain a stably integrated dioxin responsive element (DRE)-driven firefly luciferase reporter plasmid, pGudLuc6.1, that responds with the induction of luciferase gene expression in a ligand-, dose- and AhR-dependent manner [18,19,21]. Cells were plated into white, clear-bottomed 96-well tissue culture dishes at 75,000 cells/well and allowed to attach for 24 h, then incubated in triplicate with carrier solvent DMSO (1% final concentration), the indicated concentration of TCDD or the indicated mass equivalent of sediment/soil extract (the PCDD/F fraction) for 24 h at 37 °C. For luciferase measurement, treatment media was removed from the sample wells, the wells rinsed with 100 μ l phosphate-buffered saline followed by addition of 50 μ l of Promega cell lysis buffer and mixing on an orbital shaker for 20 min at room temperature

to ensure complete cell lysis. Treated 96-well plates were either analyzed immediately or stored frozen at -80°C . Measurement of luciferase activity in each well (integrated over 10 s after a 10 s delay) was carried out using either a Berthold or Anthos Lucy2 microplate luminometer with automatic injection of 50 μl Promega stabilized luciferase reagent. Luciferase activity in each well was expressed relative to that maximally induced by 1 nM TCDD after background correction. All plates contained a standard curve of at least nine concentrations, in triplicate, of TCDD (100 fM–10 nM) in DMSO, as well as all solvent controls. Each sample analysis consisted of nine dilutions of the PCDD/F fraction of each extract in triplicate, along with appropriate solvent blanks. All materials that came in contact with TCDD were handled and disposed following procedures approved by the Office of Environmental Health & Safety at the University of California, Davis.

2.4. Statistical methods

General data and statistics were performed using Microsoft Excel. EC_{50} values from full concentration–response curves of the TCDD standard and sample extractions were determined using the four-parameter Hill equation (SigmaPlot (Systat)), or for incomplete sample induction curves the EC_{50} was determined by direct comparison of results to the TCDD standard curve. EC_{25} values were determined for those sample concentration response curves where maximal luciferase activity was less than the EC_{50} of that of the TCDD standard curve, by direct comparison to the TCDD standard curve. Total relative CALUX activity of each extract was determined and expressed as pg bioanalytical equivalents (BEQs) per gram of soil/sediment.

3. Results and discussion

Three main elements are essential for the accurate determination of the relative potency (BEQs) of dl-HAHs present in an extract using the CALUX or any other AhR-based bioassay: (1) efficient extraction and clean-up procedures to isolate the desired dl-HAHs from the sample extracts with little carry over of undesired AhR agonists; (2) specific criteria establishing comparisons of sample extract and TCDD concentration induction curves; and, (3) data covering the best possible range of induction in order to establish as complete a sample extract induction curve as possible with the optimal results having both maximal and minimal plateaus to the induction curve and a slope comparable to that of the TCDD standard curve. In CALUX analysis, decreasing amounts of sample extract are added to the cells and if AhR active compounds or dl-HAHs are present, a concentration-dependent luciferase induction response curve is generated that can be directly compared to that produced by increasing concentrations of TCDD, thus allowing calculation of the relative inducing potency of the sample extract after correction for sample dilution [14,19]. While relative potency values estimated from GC/HRMS analysis are expressed as TEQ values (utilizing TEFs obtained from *in vivo* toxicity studies [10]), relative potency values from our CALUX bioassay luciferase induction results are expressed as BEQs. Use of BEQ is more appropriate and less confusing than the range of previously reported bioanalytical potency descriptors such as: TEQ, TCDD equivalents, Bio-TEQs, CALUX-TEQs, IEQs (induction equivalents) and others, especially since AhR-based bioassays do not provide a direct measure of the toxicity of dl-HAHs present in the mixture [19], but only of its potency to activate the AhR and AhR-dependent gene expression (which is mechanistically related to the adverse effects of dl-HAHs [1,2,5–10]).

While assessment of the BEQs of sediment/soil extracts by CALUX bioassay analysis has been standardized, there is signifi-

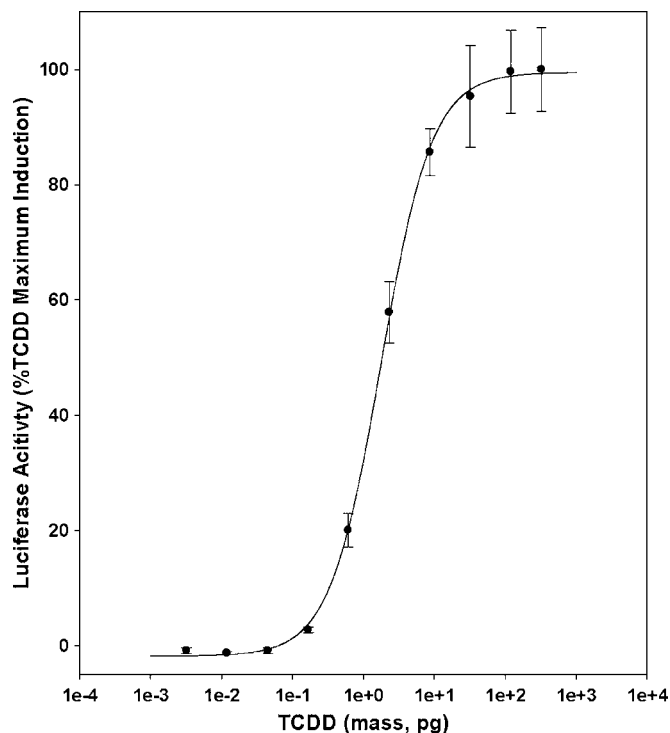


Fig. 1. Concentration–response curve for the induction of luciferase activity by TCDD in CALUX H1L6.1c3 cells. Cells were plated in 96-well plates for 24 h and incubated with the indicated concentration of TCDD (pg/100 μl incubation volume) for 24 h, followed by determination of luciferase activity as described in Section 2. Values were expressed as a percent of the maximum induction by TCDD and represent the mean \pm SD of at least triplicate determinations.

cant diversity in actual concentration–response to different sample extracts and this can impact both data interpretation and the accuracy of potency (BEQ) determinations [14,19,32,39]. Accordingly, we have examined a wide variety of sediment/soil samples by CALUX analysis in order to present the actual diversity in response observed for this assay and approaches taken to improve bioassay-based potency determination and assay interpretation. Incubation of the CALUX cells with TCDD results in a concentration-dependent induction of luciferase activity significantly above background beginning at a concentration of ~ 0.1 pg/well to a maximal activity at ~ 100 pg/well with an EC_{50} of ~ 1 –2 pg/well (Fig. 1). Analysis of the PCDD/F fraction of sediment/soil extracts in the CALUX bioassay resulted in a variety of distinct concentration-dependent luciferase induction curves with maximal or submaximal induction levels as compared to that of TCDD (compare Fig. 2 to Fig. 1). While sample 113 induced maximally, and sample 83 induced slightly submaximally, compared to that of TCDD (Fig. 2), the remaining extracts (samples 102, 112, and 118) produced concentration–response curves with the maximal induction occurring in the linear part of the induction curve, but below TCDD maximal induction thus yielding an ‘incomplete’ concentration response curve (i.e. a submaximal induction curve). The lower than maximal activity could result from low levels of AhR agonists in the sample extracts (102, 112 and 118) and the presence of AhR antagonist which reduce the overall inducing potency of the AhR agonists present in the sample. Higher concentrations of these extracts could not be evaluated because they produced cell toxicity (data not shown). These CALUX results are typical of those obtained for soil and sediment extract analysis. Standard approaches for determination of the BEQ from sample extract concentration induction curves involves using the four-parameter Hill plot to determine an EC_{50} value for the TCDD standard curve and for those sample extracts which produce a full concentration response data (i.e. whose maximal induction activ-

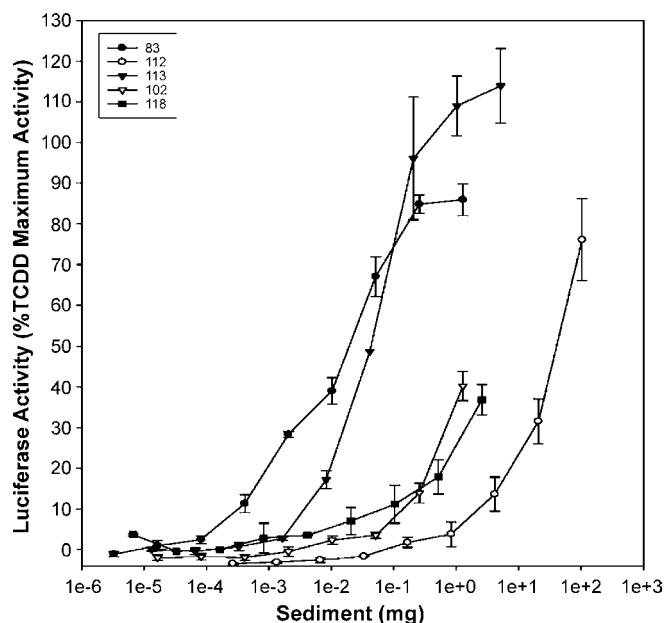


Fig. 2. Concentration-dependent induction of luciferase activity by extracts of soil/sediment in CALUX mouse H1L6.1c3 cells. Cells were plated and incubated with increasing concentrations of the PCDD/F fraction of the indicated soil/sediment extracts (mg equivalents/100 μ l incubation volume) and luciferase activity determined as described in Section 2. Values were expressed as a percent of the maximum induction by TCDD and represent the mean \pm SD ($n \geq 3$).

ity was comparable to that of TCDD such as sample 113). However, the four-parameter Hill plot cannot be used when full induction curves (with plateaus at the lower and upper end) are not achieved. For extracts with incomplete or sub-maximal induction yet whose maximal value exceeds the TCDD EC_{50} (samples 83 and 112 (Fig. 2)), direct comparison of the EC_{50} s of the samples to the EC_{50} for the TCDD standard curve (determined from a four parameter Hill plot) was used to calculate the BEQs for those sample extracts. In contrast, an EC_{25} value for TCDD derived using the four parameter Hill plot from the TCDD standard curve, was used for direct comparison for those extracts whose induction levels were $>EC_{25}$ but $<EC_{50}$ of the TCDD standard curve (samples 102 and 118 (Fig. 2)). The results of these determinations are summarized in Table 1 along with the GC/HRMS-derived TEQ values for these samples calculated using the WHO 2005 TEF values and previous GC/HRMS results [38]. Evaluation of the BEQ/TEQ ratio revealed that while CALUX analysis

was a reasonably good predictor for PCDD/Fs in sample 112 (with a 2-fold higher BEQ value), the BEQs for the remaining sediment samples was between 7- and 266-fold higher than that determined by GC/HRMS. It is likely that the higher BEQ potency estimate for sample 83 compared to its TEQ value (266-fold higher) results from the fact that the slope of its induction curve was not parallel to the TCDD standard curve (or that of samples 112 and 113), suggesting some modulation (enhancement) of the AhR induction response by another chemical(s) present in the extract. Given the uncertainty in BEQ estimates from nonparallel curves, it has been proposed that a range of potency values be determined using various points on the induction curve in order to better describe the resulting potency estimate. The elevated BEQ activities of each of the first test set of samples (83,102,112,113,118), relative to the GC/HRMS TEQ values, suggests that these extracts contain AhR agonists in addition to the PCDD/Fs. Potential chemicals could include brominated- or mixed chloro/bromo-dibenzo-p-dioxins, dibenzofurans, chloro-naphthalenes and others that have been shown to be AhR active [31,32,39–42], but are not currently measured using standard GC/HRMS analysis and currently do not have TEF values.

Interestingly, concentration-dependent CALUX screening analysis of other sediment and soil extracts revealed many sample extracts that produced superinduction of luciferase activity to between 1.5- and 4.5-fold greater than that maximally induced by TCDD (Fig. 3). While a superinduction response is not entirely surprising given that we have previously observed this effect using crude soil and sediment sample extracts that have not undergone any chemical cleanup step to isolate dl-HAHs (data not shown). The fact that a “cleaned-up” PCDD/F extract fraction can produce this enhanced response suggests that it must contain compounds capable of dramatically enhancing the AhR-dependent CALUX induction response since superinduction by purified mixtures of PCDD/Fs have not been reported. Although superinduction of AhR-dependent gene expression has been previously observed by our laboratory and others [20,33–37], the exact chemical(s) and/or molecular mechanisms responsible have not been elucidated, but several different mechanisms have been proposed. Inhibition of AhR degradation by proteolysis has been shown to increase intracellular levels of ligand (TCDD)-activated AhR and this has been proposed to increase the magnitude of AhR-dependent gene expression [43,44]. A labile repressor protein has also been proposed and inhibition of expression and/or enhanced degradation of this repressor would result in an increase in AhR functionality and enhanced transcriptional response [36,43]. Assuming these mechanisms to be responsible for the sediment and

Table 1

Summary of CALUX BEQ and GC/HRMS TEQ determination for extracts of soil and sediment. Soil and sediment samples were extracted and analyzed using standard methodology by CALUX and GC/HRMS. For extracts that superinduced in the CALUX bioassay (SI-F and SI-SM), each concentration–response curve was normalized to its maximal induction level (set at 100%) and EC_{50} values for each curve determined as described in the text.

Sample no.	GC/MS TEQs (pg/g sed)	Curve type ^a	Standard analysis			Normalization	
			BEQ (EC_{50}) (pg eqv/g)	BEQ (EC_{25}) (pg eqv/g)	BEQ/TEQ	BEQ (n EC_{50}) (pg eqv/g)	BEQ/TEQ
83	229	F	60918	ND ^b	266	NA ^c	NA
102	16	S50	NA	864	54	NA	NA
112	13	SM	24	ND	2	NA	NA
113	502	F	28317	ND	56	NA	NA
118	68	S50	NA	484	7	NA	NA
94	43	SI-SM	7679	ND	179	896	21
95	8582	SI-F	43309	ND	5	8726	1
96	8397	SI-F	70985	ND	8	7680	0.9
97	10968	SI-F	57654	ND	5	7472	0.7
98	10773	SI-F	71244	ND	7	8500	0.8
115	13	SI-SM	958	ND	74	275	21

^a Full curve (F), submaximal (SM), sub- EC_{50} (S50), superinduction full curve (SI-F), superinduction submaximal (SI-SM).

^b Not determined.

^c Not applicable.

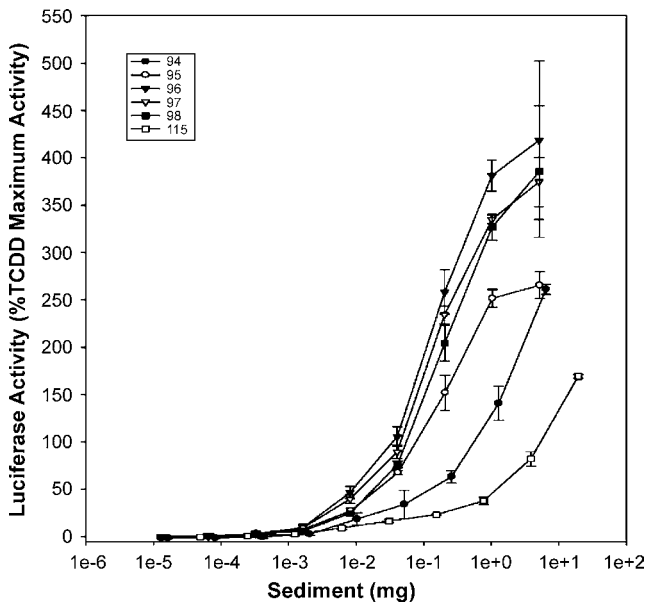


Fig. 3. Superinduction of luciferase by extracts of soil/sediment in CALUX mouse H1L6.1c3 cells. Cells were plated and incubated with increasing concentrations of the PCDD/F fraction of the indicated soil/sediment extracts (mg equivalents/100 μ l incubation volume) and luciferase activity determined as described in Section 2. Values were expressed as a percent of the maximum induction by TCDD and represent the mean \pm SD ($n \geq 3$).

soil extract superinduction response, these extracts would need to contain a chemical(s) that would inhibit synthesis of the repressor and/or inhibit cellular proteolytic mechanisms that degrade the AhR. Although these mechanisms of superinduction are possible, they are unlikely as these extracts do not produce the toxicity associated with protein synthesis or proteosomal inhibitors. While one might envision that it also may be possible that the superinduction response might result from a compound(s) present in these extracts which has a higher affinity and greater inducing potency than that of TCDD, no such compound has ever been identified and it is well documented that TCDD is the most potent AhR agonist

[1,2,10]. A more likely explanation is that in addition to containing dioxin-like HAHs that may co-activate or directly activate the AhR, the extracts also contain a chemical(s) that affects other cellular signal transduction pathways augmenting the induction response. In fact, support for this hypothesis comes from the observation that activation of the protein kinase C signaling pathway as well as treatment of CALUX cells with selected prostaglandins, known signaling factors, results in a synergistic increase in TCDD-inducible, AhR-dependent gene expression [20,33–37,43,44].

The concentration-dependent superinduction response resulting from a select number of these extracts (Fig. 3) revealed that luciferase induction reached a maximal level and plateau with four of the samples (#95, 96, 97 and 98), while that with samples 94 and 115 failed to reach a maximal level plateau. Similar to the previous results in Fig. 2, it is possible that the incomplete concentration response induction curve derives from the presence of relatively low levels of AhR agonists in the sample extracts and/or the presence of AhR antagonists that reduce the overall inducing potency of the AhR agonists present in the sample. However, the chemical(s) responsible for the superinduction response would still enhance the magnitude of induction in either situation. In contrast to the previously established approach to determine the relative potency (BEQ) of the sample extracts shown in Fig. 2, estimation of the BEQs from superinduction curves is problematic as these results are not directly comparable to the TCDD standard curve. For example, using the standard CALUX analysis method to directly compare luciferase activity at the EC_{50} for TCDD to the same amount of luciferase activity of the superinduced samples is shown in Fig. 4 (compare points A1 to B1 and C1). Calculation of BEQs using the standard analysis approach and comparing the values to the GC/HRMS TEQs determined for the same samples (Table 1) reveals that the BEQ potency values of the superinduced samples are overestimated by a factor of 5–179-fold. Given our understanding of some of the mechanisms of superinduction of AhR signaling, it is highly likely that the superinduction response is chemical concentration dependent and occurs proportionately at all extract concentrations and as such, the superinduction response would remain directly proportional to TCDD standard induction. Accordingly, a more valid approach for relative potency calculation from superinduction data when a

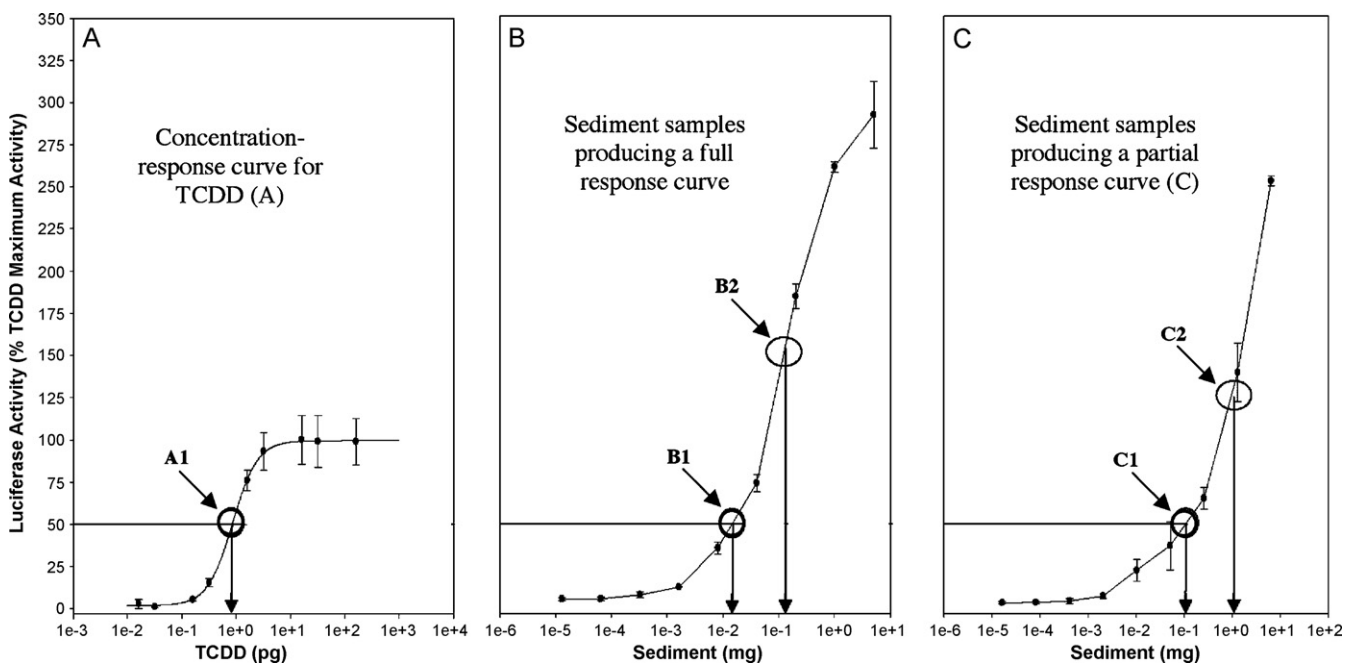


Fig. 4. Approach to determination of relative potency values (EC_{50}) values for soil/sediment samples producing superinduction in the CALUX bioassay (see text for details).

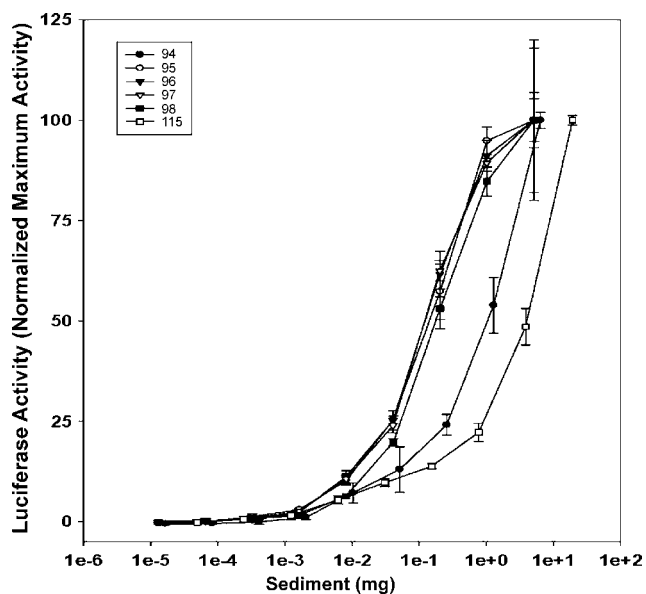


Fig. 5. Normalization of concentration-dependent superinduction of luciferase by extracts of soil/sediment in CALUX mouse H1L6.1c3 cells. CALUX induction results for soil/sediment extracts in Fig. 3 were each normalized to 100% of their respective maximal induction level and expressed as the mean \pm SD ($n \geq 3$) of the percent of the maximal value.

full concentration–response curve is obtained, is to set the maximal induction (i.e. the upper plateau) of the superinduction curve at 100% response and determine the 50% response (EC_{50}) for each sample curve and then calculate the appropriate BEQ value using the TCDD EC_{50} from the standard curve (compare points B2 and C2 to A1 for TCDD (Fig. 4)). This normalization results in a significant decrease (~ 10 -fold) in the overall relative sample extract potency (BEQ value) of the superinducing sample extract compared to that estimated by direct comparison of results with the EC_{50} of the TCDD standard curve (Fig. 4, compare points B1 to B2). Alternatively, for visual clarity and comparative purposes (both for potency and to confirm parallel induction responses), the results of each curve can be normalized to its own maximal activity and all of the results plotted together (Fig. 5). Comparison of the BEQ values for the four superinducing sediment samples that produced full concentration response curves (samples 95–98) before and after normalization (Table 1), not only revealed that normalizing the results of these curves decreased their BEQ values by a factor of 5–9-fold, but the resulting BEQ values were now nearly identical to the GC/HRMS TEQ values (BEQ/TEQ ratio of 0.7–1). Thus, internal normalization of CALUX superinduction results from full concentration–response curves allows increased accuracy and precision of relative potency estimates of PCDD/Fs in sample extracts.

Full concentration response curves are not obtained for most sample extracts run in the CALUX bioassay and while this can complicate BEQ estimations from these types of data, the accuracy of the BEQ estimate depends on the magnitude of the induction response obtained (i.e. do the maximal results fall in the linear working range and/or are they greater than 50% of the maximal TCDD induction response). This is even more problematic when attempting to calculate BEQs from superinduction results of concentration response curves of submaximal induction curves (i.e. those that do not reach an upper plateau (Fig. 4C)), since the maximal response we would set to 100% is unknown. Accordingly, the most conservative approach for BEQ estimation from submaximal superinduction results would be to set the highest point of the induction curve as the maximal induction (i.e. 100% response) and arithmetically determine the 50% value (EC_{50}) of the induction curve (Fig. 4C). The adjusted decrease in extract potency is demon-

strated by a nearly 10-fold increase in EC_{50} potency value, compare points C1 and C2 in Fig. 4, and while it is acknowledged that there may be some overestimation of the overall sample extract potency, the resulting BEQ value from this internal normalization is closer to the analytical toxic potency (i.e. the TEQ). Normalization of the submaximal superinduction results to 100% response also allows them to be directly compared to all other CALUX concentration–response curves (Fig. 5). Direct comparison of the effect of normalization of submaximal superinduction data (samples 94 and 115) reveal that normalization significantly decreases the CALUX BEQ potency estimate and increases the accuracy of the BEQ estimate of the PCDD/F TEQ values for these sample extracts by 8–13-fold (i.e. reducing the standard analysis BEQ/TEQ ratio for samples 94 and 115 from 179 and 74, respectively, to 21 for both samples with normalization (Table 1)). The lack of a complete induction curve for the submaximal superinducing sample extracts certainly contribute to the remaining overestimation of the potency of these samples, although the impact of other factors such as the presence of additional AhR agonists in these extracts cannot be discounted. Taken together, our results demonstrate that normalization of sample extract CALUX superinduction results provides a valid approach for more accurate determination of BEQs of dl-HAHs in sample extracts and should be used when such a response is obtained.

The improved correlation between TEQs and normalized superinduction BEQs, coupled with our relatively common observation of CALUX superinduction by soil and sediment samples, suggests that evaluating soil and sediment extract potencies by simple direct comparison to the EC_{50} of the TCDD standard curve without full concentration–response analysis can contribute to overestimated CALUX-based BEQ values. Superinduction could even contribute to BEQ overestimation for those sample extracts that do not induce to 100% of that maximally induced by TCDD. In this instance, the presence of “superinducing” compounds in the sample extract could enhance the magnitude of response to a low level of AhR-active dl-HAHs, thus making the sample appear more potent than it actually is and resulting in an overestimated BEQ value. However, until the mechanisms responsible for the superinduction responses by these sediment/soil and other extracts are elucidated, the contribution of superinduction to BEQ potency estimates from submaximal induction curves cannot be determined. Taken together, our results not only report for the first time the phenomenon of superinduction of the CALUX bioassay by “cleaned-up” PCDD/F fractions of sediment and soil extract, but we also provide an avenue by which normalization of such data leads to more accurate potency estimates. In addition, they demonstrate that for optimal and most accurate BEQ determination for all soil, sediment, or environmental extracts by CALUX and likely other AhR-based bioassays, whenever possible, full concentration response curves should be utilized.

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