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# Chemically Activated Luciferase Gene Expression (CALUX) Cell Bioassay Analysis for the Estimation of Dioxin-Like Activity: Critical Parameters of the CALUX Procedure that Impact Assay Results

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The *c*hemically *a*ctivated *luc*iferase gene e*x*pression (CALUX) in vitro cell bioassay is a bioanalytical tool that is increasingly being used by research and commercial laboratories for the screening and relative quantification of dioxins and dioxin-like compounds in sample extracts. Since CALUX analyses provide a biological response to all aryl hydrocarbon receptor active compounds present in a given sample extract containing a complex mixture of chemicals, interpretation of results is significantly more complex than of chemical analyses. Operators in the laboratory can adjust many parameters when performing CALUX analyses, and the applied procedure strongly affects the result and, hence, the interpretation of the results. This paper examines critical methodological parameters and aspects of the CALUX bioassay that can affect the quality and accuracy of the analyses. Moreover, the study aims to identify the ways that alteration of these parameters influences CALUX measurements. A greater understanding of these characteristics will lead to increased accuracy, precision, and reproducibility of the widely used CALUX bioassay within and between research laboratories.

## Introduction

The *c*hemically *a*ctivated *luc*iferase gene e*x*pression (CALUX) in vitro cell bioassay is a bioanalytical tool that is increasingly being used by research and commercial laboratories for the screening and relative quantification of dioxins and dioxin-like compounds in blood (*1*−*4*), sediments (*5*−*8*), food matrices (*9*−*12*), and milk (*13*, *14*) (see refs *15*−*18* for reviews). This screening and prioritization tool, cheaper and more rapid than conventional instrumental chemical analysis of dioxins, is mandatory for the analysis of the high number of samples required for food and feed safety (*19*) and to monitor and protect the environment.

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CALUX is a reporter-gene-based cell bioassay that uses genetically modified cells that respond to chemicals that activate the aryl hydrocarbon receptor (AhR). The AhR is a ligand-dependent transcription factor that not only binds and is activated by dioxins and related chemicals but is also responsible for mediating the toxicity of these chemicals. A common response resulting from activation of the AhR signaling pathway in cells is the induction of gene expression, and this forms the basis of this bioassay system. The recombinant cells used in the CALUX bioassay contain a stably transfected AhR-responsive firefly luciferase reporter gene that responds to dioxins and any other chemical(s) that can bind to and activate the AhR, leading to the induction of luciferase gene expression. Induction of luciferase in the recombinant CALUX cell line occurs in a time-, dose-, and AhR-dependent and chemical-specific manner, and the amount of induced luciferase activity is directly proportional to the amount and potency of the inducing chemical (i.e. AhR agonist) to which the cells have been exposed (*20*, *21*). The measured luminescence resulting from exposure to a chemical or chemical mixture is converted into a bioassay toxic equivalency (CALUX-TEQ) value by the direct comparison of the response for a given sample to a dose−response curve obtained with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin). The AhR mechanism and the CALUX bioassay system have been described in detail (*18*, *21*, *22*).

Since CALUX analyses provide a biological response to all AhR active compounds present in a given sample extract containing a complex mixture of chemicals, interpretation of results is significantly more complex than of chemical analyses. Operators in the laboratory can adjust many parameters when performing CALUX analyses, and the applied procedure strongly affects the result and, hence, the interpretation of the results. There is a large number of laboratories using this technology, there are a variety of CALUX-type cell bioassays, and while some methods have been described by the major research groups, there is no commonly accepted method for this analysis. In addition, there have been no publications that have systematically evaluated of the influence of each aspect of the CALUX methodology on the quality of the resulting analysis. This is a key issue given that comparisons of data from different laboratories are not possible, since significant differences in

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analytical and assay methodologies exist between laboratories. In an effort to overcome these difficulties, commercial suppliers of the CALUX cell lines have proposed standard operation procedures (SOP) for different matrices. While intercalibration studies between the different labs reveal that assay results of identical samples (pure chemicals or crude samples) from within and between laboratories are on the same order of magnitude, significant differences in the reproducibility and repeatability were also observed (*23*, *24*).

However, even if most of the parameters for the CALUX protocol are described in the SOPs provided by the supplier, the reasoning and impact of the choice of these parameters on the results and their interpretation are commonly not included. When we consider that many other laboratories are also utilizing CALUX bioassays with their own sample preparation and assay protocols, understanding the impact that variations in the assay methodology have on the quality of the resulting CALUX data is of significant importance. In addition to methodological issues, several fundamental questions about the CALUX bioassay, its application, and its utility remain to be established or defined. For example, can the measured CALUX reporter gene induction response be considered as an overall TEQ value and, hence, as a proxy of the sample's dioxin-like toxicity? Can the response correctly approximate the results of chemical determination of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (PCBs) in sample extracts? Are results obtained from both analytical and bioanalytical approaches similar only when analyzed under well-controlled conditions? Do we need to analyze all AhR ligands or just the dioxin-like compounds? What are the similarities and differences between these chemicals with regard to their biological and toxicological potency?

The initial objective of this paper is to summarize information on the critical methodological parameters of the CALUX bioassay that can affect the analyses results. Moreover, our study aims to identify the ways that alteration of these parameters influences CALUX measurements and attempts to interpret the CALUX response. This paper will examine all steps of the analytical/bioanalytical procedures, including (1) extraction and sample cleanup, (2) exposure of cells to the analytes, and (3) data handling and interpretation. The analysis of one sample of sediment (Rupel sediment) using different parameters will be used to illustrate the importance of analytical procedures on the overall results and their interpretation (the added new data are illustrative in nature and not necessarily meant to be quantitatively reproducible). This matrix type was chosen since analysis of the raw extract is possible and since many classes of compounds are present in this complex matrix. Other matrices are not investigated but would most assuredly lead to similar conclusions.

## Discussion

**Extraction.** The first step in the analysis is the extraction of dioxins and dioxin-like compounds from their matrix. Conceptually, CALUX analyses provide an overall response for all compounds present in the extract that are able to activate the AhR and AhR-dependent gene expression. Therefore, the specific extraction conditions employed will determine the number and chemical properties of extracted chemicals as well as their respective percentages of recovery.

Many different chemicals found in extracts are able to stimulate AhR-dependent gene expression. They have been subdivided into "classical" and "nonclassical" AhR ligands and inducers by Denison et al. (*26*, *27*). Classical AhR ligands are defined as planar, aromatic, and hydrophobic compounds, with a molecular structure having characteristics similar to that of TCDD. However, some classical and strong AhR inducers, such as polycyclic aromatic hydrocarbons (PAH), are not considered as dioxin-like compounds, since they do not meet the criteria of the toxic equivalent factor (TEF) approach (*28*) (i.e. they do not produce AhR-dependent toxic effects). These criteria imply that dioxin-like compounds are structurally related to dioxins, biologically and environmentally persistent, work through the Ah receptor, and cause the same spectrum of responses. Other compounds with physicochemical and structural properties dramatically different from that of TCDD are termed nonclassical AhR ligands, and for the most part, they are moderate to weak AhR inducers (*26*, *27*). These nonclassical AhR ligands do not produce AhR-dependent toxicity, and as such, they do not meet the criteria of the TEF approach (*28*).

The list of the already identified Ah ligands is long. A review on classical AhR ligands was published by Behnisch et al. (*15*). The authors list the relative potencies (REP) of some AhR agonists determined using different bioanalytical methods and they provide a diagnosis of the pollutants' sources, fates, and levels in the environment. Recent studies have provided additional information about the wide range of AhR agonists and antagonists that have been identified and these studies have revealed that the AhR can be bound and activated by numerous chemicals and chemical classes with little structural similarity and physiochemical characteristics distinct from TCDD and other classical AhR agonists (*26*, *27*, *29*–*32*). The diversity in physiochemical characteristics of AhR agonists and antagonists complicates the cleanup process designed to eliminate other AhR agonists from TCDD and related dioxin-like halogenated aromatic hydrocarbons (HAHs).

In biotic samples, dioxins and most of the classical hydrophobic AhR ligands are primarily distributed in lipid-rich tissue and are usually easily extracted from tissues using organic solvents. For abiotic samples, the extraction conditions raise the question of bioavailability, a problem that is posed for the chemical analysis as well as for bioassay. Until now, it was generally assumed that the extraction technique yielding the highest concentration of the compounds of interest is the best. However, only a fraction of the compounds of interest may be available to environmental processes such as transport and biological uptake (*33*). This fraction can be extracted using soft extraction conditions. Extraction of the more tightly bound fraction requires stronger extraction conditions (*34*–*37*). When interpreting data, one should keep in mind that toxicological interpretation should not be based only on concentration but also on the bioavailability of the compounds of interest.

**Consideration in Sample Cleanup.** The cleanup method for samples predominantly controls the response and/or TEQ measurement and is a major parameter for CALUX.

*1. Fractionation Approaches for Isolation of Desired Halogenated Aromatic Hydrocarbons.* For some sample matrices, the resulting crude extract can be analyzed directly by CALUX, without any cleanup. However, in this case, every chemical that exhibits AhR agonist/antagonist activity that is present in the extract will contribute to the overall CALUX's response in the cell line. While the lack of any induction response by an extract would suggest the absence of any AhR agonist in the sample [i.e. the absence of the chemicals of concern (TCDD and related chemicals)], the presence of antagonists and/or chemicals that are toxic to the cells could be responsible for the negative response. The presence of AhR antagonists in the sample can be readily determined by examining the ability of the extract to inhibit the response of the cells to TCDD and the presence of chemicals toxic to the cells can be readily determined by assessing the health of the cells using cell viability assays. Thus, as a rapid screen, the CALUX assay could be used to assess the absence of the chemicals of interest. However, determination of the presence of dioxin-like HAHs in a positive sample extract requires

**TABLE 1. Concentrations in pg CALUX-TEQ/g of Sediment of Selected PAHs in the Extract of the Rupel Sediment, Calculated by Multiplying the Concentration Determined by Chemical Analysis by the REP Comparison with the Concentrations Measured in CALUX for the Extract (24-h Incubation)**

| | recalculated concentration | | | | | | CALUX measurement of the extract (pg CALUX-TEQ/g of sediment) |
|---|---|---|---|---|---|---|---|
| cleanup | benzo[*a*]-anthracene | benzo[*b*]-fluoranthene | benzo[*k*]-fluoranthene | benzo[*a*]-pyrene | dibenzo[*a,h*]-anthracene | sum of 5 PAH | |
| crude extract | 55 | 1688 | 982 | 197 | ND | 2923 | 4500−14400 |
| acidic silica + carbon, dioxin fraction | ND | 100 | ND | ND | ND | 100 | 80−105 |
| extended cleanup, dioxin fraction | ND | ND | ND | ND | ND | ND | 18−30 |
| REP ($EC_{20}$) (*40*) | 0.00015 | 0.0038 | 0.0045 | 0.00052 | 0.0039 | | |

application of a cleanup procedure to isolate the chemicals of interest and to confirm their activity in a subsequent CALUX analysis. Generally, screening of whole extracts is not done, due to the ubiquitous nature of AhR agonists in environmental, biological, and food samples. For most sample extracts, a cleanup procedure is applied that removes the matrix component, but this also results in removal of some AhR ligands from the final extract that will be analyzed. Acidic silica columns are usually used in the CALUX methodology (acidic silica:~30% $H_2SO_4$ (w:w)), which aids in the removal of fat and some other interfering compounds from the crude extract. Under these acidic conditions, most of the nonclassical AhR ligands and some classical AhR ligands (i.e. PAHs) are readily separated from the desired HAHs and consequently do not contribute to the final response. To separate PCDD/F from most of the other compounds and improve the selectivity of CALUX analyses, a carbon column can be added in series with the acidic silica column (*9*, *12*, *14*, *25*, *38*, *39*). In this case, the response of the CALUX analysis for the different fractions is directly related to the efficiency of the column to separate the different AhR ligands.

Table 1 illustrates the impact of the cleanup on CALUX results obtained for the Rupel sediment after 24-h incubation of the extract. The concentrations measured in pg TEQ/g of sediment for the crude extract (4500−14400 pg TEQ/g) are about 140 times higher than for the extract cleaned with an acidic silica column + carbon column (dioxin fraction) and about 500 times higher than for the extract cleaned with a multilayer acidic silica/silver nitrate/alumina column + carbon column (dioxin fraction). When the crude extract is analyzed, the very high response measured is most likely due to the presence of high concentrations of PAHs. To evaluate the order of magnitude of the contribution of PAHs to the response, the concentrations of five PAHs in Rupel river sediment were determined (Table 1) by chemical analysis. As REP values for these PAH are not currently available under the conditions used in this study, these concentrations were multiplied by the CALUX REP determined in rat H4IIE cells after 24-h incubation (based on the $EC_{20}$) (*40*). The REP values for PAHs are expected to be on the same order of magnitude in both cell lines, as for PCDD/F and PCB (*12*, *40*, *41*). A concentration of 2923 pg CALUX-TEQ/g of sediment was calculated for this sample and it is on the same order of magnitude as the concentration measured for the crude extract by instrumental analysis (4500−14400 pg CALUX-TEQ/g). As only five PAH were considered and as it is likely that additional PAHs and nonclassical AhR ligands are present, it can be concluded that the majority of the CALUX response in the crude extract is due to PAH.

When the crude extract is cleaned with an acidic silica column + carbon column, only 6% of the benzo[*b*]fluoranthene remains in the cleaned extract. Even this small amount of one PAH gives a CALUX response (100 pg TEQ/g) on the same order of magnitude as that of PCDD/Fs (18−30 pg TEQ/g). To eliminate all PAHs and nonclassical AhR ligands from a sample extract such that PCDD/Fs can be analyzed more selectively, a more extensive cleanup, such as a multilayer acidic silica/silver nitrate/alumina column and carbon column, is mandatory.

*2. Toxicity of Solvents and Extracts.* In addition to the chemicals present in the samples, impurities of sample preparation solvents and adsorbents can significantly affect the CALUX measurement. Some compounds can lower or completely suppress the CALUX response as a result of their ability to cause cell toxicity and death, inhibition of AhR-dependent induction of gene expression (which can occur by many possible mechanisms, including the presence of AhR antagonists in the sample extract), inhibition of luciferase enzyme activity, increased degradation of the luciferase reporter enzyme, and other mechanisms. Consequently, great attention must be paid to the quality of the solvents and adsorbents used, and their potential impact has to be tested.

The presence of these interfering compounds can be checked by using a double control test, as suggested by Windal et al. (*12*). In this instance, a procedural blank is performed, using the same adsorbents and solvents as the samples, and the final extract is spiked with a standard solution of TCDD. The standard solution and the spiked extract are then analyzed in the same way. There should be no significant difference between both results. For responses that are greater than that of TCDD alone, it can be concluded that the procedure introduces AhR ligands and/or enhances AhR-dependent gene expression by other methods. On the other hand, responses that are lower than that of TCDD alone suggest that the solutions contain a chemical(s) that reduce the induction response or luciferase activity of the lysed cells, as described above.

When procedural blanks are directly analyzed (without the addition of the standard solution of dioxin), a positive CALUX response indicates contamination. However, a CALUX response equal to zero would be observed when there is no contamination but also when some interfering compounds present in the procedural blank would inhibit the induction response or reporter gene activity. With the procedure proposed here, compounds that increase and/or decrease the cell's induction response can be readily detected.

For example, in our lab, when just one column of acidic silica is used for the cleanup, the response of the spiked procedural blank is only about half of the expected response. Results of the Rupel sediment using this cleanup procedure are then lower than they should be, since the response of the extract is reduced as a result of some compound(s) introduced by the procedure. Accordingly, no clear interpretation of the results can be made.

*3. Synergetic and Antagonistic Effects of the Compounds Present in the Purified Extract.* The TEF principle used in chemical analysis assumes the additive contribution of the different PCDD/F and non- and mono-ortho PCB (*28*). However, important nonadditive interactions between AhR

ligands have been described and are measured, at least in part, by CALUX. The nonadditive interactions only occur with compounds that are not pure AhR ligands. Studies on nonadditive effects of HAH mainly focus on individual polychlorinated biphenyls (PCB 52, 108, 153, 156, 159) or Aroclor 1254 (see ref *42* for a review), which are able to inhibit the biological response induced by TCDD. Strong antagonistic effects are also observed for some other compounds, such as hexachlorobenzene (*25*). When high concentrations of these compounds are present in an extract, the CALUX response due to the PCDD/F may be almost completely masked.

Synergistic interactions, on the other hand, have been less frequently observed and can occur at much lower dose ratios. These effects have been observed between AhR ligands and chemicals affecting other signal transduction pathways. One example is reported with corticosteroids, which alone induce a very weak response in CALUX but can dramatically enhance the response of TCDD in rat hepatoma (H4IIe) cells. This may result from an indirect effect resulting from an increase in the concentration of the AhR (*31*) or activate the AhR/AhR-dependent gene expression in a cell- or species-specific manner. Transient synergism in AhR signaling has also been observed with prostaglandins (*43*) and activators of protein kinase C (*44*). Synergism has also been observed for several responses in vivo, such as porphyrin induction and effects on thyroid hormones (*45*).

Since some of the AhR agonists and antagonists are discarded during the extraction and cleanup procedure, only a fraction of them is actually analyzed in the assay. This also means that only part of the nonadditive interactions are measured by CALUX. CALUX measurements are then not necessarily closer to the "real dioxin-like activity of the sample" than chemical analysis, which cannot consider any nonadditive interaction.

**Analysis.** *1. The Importance of Cell Lines.* CALUX analyses can be performed with many different cell lines, the main limitation being that they must contain a fully functional AhR signaling pathway. However, it should be noted that results obtained with different cell lines can vary significantly for two primary reasons: the first is related to species- and tissue-specific differences in the relative concentration and functionality of the AhR, its functional subunits (including its DNA binding partner, the Ah receptor nuclear translocator (Arnt) as well as nuclear coactivators, transcription factors, and cofactors necessary for AhR signal transduction (*20, 44, 46–52*). This suggests that REP values of different AhR agonists vary according to the tissue and species from which the cell line used to generate the recombinant CALUX cell line was derived. The TCDD responsiveness (i.e. $EC_{50}$) of a human hepatoma (HepG2) CALUX-type cell bioassay was reported to be about 10-fold lower than that of rodent cell lines, and this likely derives from the fact that the AhR ligand binding affinity in this human line is about 10-fold lower than that of the rodent cell lines. Numerous CALUX-type cell lines have been developed by a variety of laboratories, and their relative sensitivities and induction characteristics have been tabulated in a recent review by Denison and co-workers (*18*). Second, the antagonistic and synergistic effects of some compounds are cell line dependent. For example, Garrison et al. (*20*) reported that luciferase induction by TCDD was not affected by the presence of 22′55′ TCB (PCB 52) for guinea pig and human cell lines, whereas it was lowered by a factor of 2 for a rat cell line and by a factor of 5 for a mouse cell line. These species-specific differences in responsiveness to PCB 52 were determined due to distinct differences in the AhR (*53*). Other species specific differences in AhR ligand binding specificity and functional activity have also been reported (*30*) and this will likely contribute to variability between species-specific CALUX bioassays.

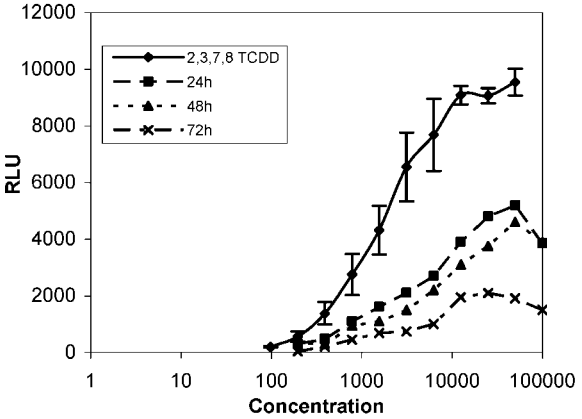


**FIGURE 1. Impact of the incubation time on the shape and the relative position of the dose–response curves measured for the crude extract of the Rupel sediment, compared to the dose–response curve of the reference, TCDD. Concentrations expressed in fg/well for TCDD and in $1.5 \times 10^{-5}$mg sediment/well for sediment samples.**

*2. The Effect of Exposure Duration.* The duration of exposure can greatly influence the CALUX measurement. Compounds such as PAHs and most of the nonclassical AhR ligands can only be measured after a short incubation. Following longer exposures, the responses to these compounds are reduced, as they are metabolized. (*7, 28, 49–51*). The duration of exposure is therefore chosen according to the objective of the measurement. For illustration, the crude extract of the Rupel sediment was analyzed after 24, 48, and 72 h (Figure 1). A complete dose–response curve cannot be generated, since toxicity at the higher concentrations examined led to a decrease of the induction response. These data are also quite difficult to interpret, since the dose–response curves are not parallel, as described below. When the same dilution of the extract is considered, a reduction of about 20% of the dioxin-like toxicity is measured when the duration of exposure is increased from 24 to 48 h and a reduction of about 70–80% is measured when the duration of exposure is increased from 24 to 72 h.

**Data Interpretation.** *1. Variation of the Response with the Dose Analyzed.* When analyzing an unknown sample by CALUX, a calibration curve is drawn with TCDD standard solutions (induction response versus log concentration of TCDD), and the best equation fitting the curve is established. The concentrations of the unknown samples, expressed as pg CALUX-TEQ/g of sample, are derived from this equation with the sample's response. However, this approach assumes that the extract analyzed behaves like a diluted or concentrated solution of the standard. This implies that the dose–response curves of the sample and of the standard are parallel and that the maximal achievable response (efficacy) for the standard and sample are identical. When these assumptions are not true, the concentrations measured are a function of the dose analyzed. Ideally, a range of concentrations should be reported instead of a single value. Numerous approaches have been previously described for comparison of parallel and nonparallel dose–response curves with regard to AhR-mediated induction of gene expression for estimation of TEQs, and the reader is referred to these reviews for a more in-depth discussion of these issues (*17, 57*).

In practice, during screening analysis only one dose is usually tested, and as such, these assumptions are not checked; thus, the concentration measured for the sample may vary with the dose used for the analysis. The degree of variation is correlated to the degree of deviation from parallelism between sample and standard dose–response curves (*55, 58*). To limit this possible bias of the measurement, the acceptable responses in CALUX should be set to less

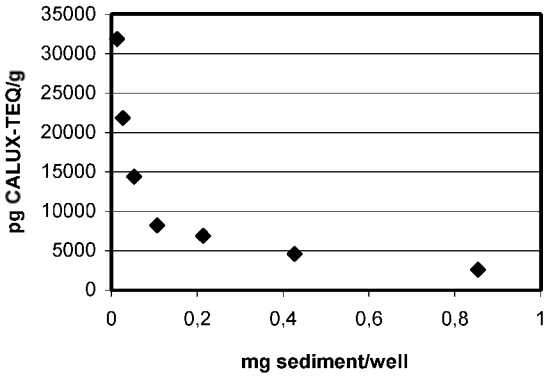


**FIGURE 2. Variation of the concentration measured by CALUX as a function of the amount of sediment analyzed.**

than half the maximal response for the TCDD standard curve (*41*). Results must be interpreted with caution when only one measurement is performed, and multiple dilutions of a sample should be measured to calculate a more reliable range of CALUX-TEQ concentrations.

The dose−response curve of the crude extract of the Rupel sediment analyzed after 24-h incubation is not parallel to the dose−response curve of the TCDD (Figure 1). The results obtained for the different dilutions, expressed as pg TEQ/g, are plotted as a function of the amount of sample used (mg of sediment/well) in Figure 2. The result for the highest amount of sample presented in the chart can be discarded, since the extract may be toxic (as it is for higher amounts). Results for the other measurements vary between 4500 and 31800 pg CALUX-TEQ/g.

To reduce this interval, quantification limits have to be considered. When setting quantification limits in our lab, three factors are taken into account: (1) the dose−response curves of some dioxin-like compounds do not reach the same maximum as the TCDD dose−response curve, (2) the antagonistic effect of some compounds is lower when working at lower concentration (data not shown), and (3) quantification at concentrations below our seventh point of calibration (781 fg/well) is less precise (*12*), since the point is closer to the noise. Consequently, it was decided to quantify only the samples giving results in the lower half of the calibration curve (concentration <3125 fg/well) and above the concentration of 781 fg/well. If only results obtained in these limits are considered, the concentrations measured for the sediment vary between 4500 and 14400 pg CALUX-TEQ/g.

*2. Determination of the Percentage Recovery.* After the measurement by CALUX, results can be corrected by taking into account the percentage recovery of dioxins during the sample preparation procedure. The determination of the percentage recovery can be done in different ways: (1) An additional sample is spiked with [$^{14}$C]TCDD. The amount of [$^{14}$C]TCDD in the cleaned extract is compared to the amount of [$^{14}$C]TCDD spiked to determine the percentage recovery. (2) An additional blank sample is spiked with TCDD or a mixture of dioxins. To determine the percentage recovery, the response of the cleaned extract is compared to the response of TCDD or the mixture of dioxins used. These methods of determination of the percent recovery suppose that the sample preparation procedure is very reproducible, so that the percentage recovery of samples analyzed in the same series are the same. When only TCDD is used, it is also supposed that the behavior of TCDD is representative of the behavior of the AhR ligands in the samples. In chemical analysis, this problem is solved by the use of labeled dioxins as internal (or recovery) standards. The losses during the sample preparation are the same for native and labeled dioxins, so that the measurement (based on ratio between native and labeled dioxins) is the same whatever is the percentage recovery.

**Analysis of the Dioxin-Like Activity in the Rupel Sediment by CALUX.** Many parameters can be chosen when performing CALUX analysis, which can greatly influence the results. For the Rupel sediment sample used in this paper for illustration, results vary from 18 to14400 pg TEQ/g according to the parameters chosen. The interpretation of the results has, therefore, to be considered carefully and related to the analytical procedure.

**Comparison of Data from Different Laboratories.** The paper of Besselink et al. (*23*) describes the results of an intra- and interlaboratory validation study (ring test with six participants) of pure chemicals and sediment samples using an identical CALUX methodology. This study concluded that the average interlaboratory repeatability was 14.6% for the analysis of pure compound and 26.1% for the analysis of whole matrix, while interlaboratory reproducibility was 6.5% for pure compound and 27.9% for whole matrix. However, a closer look at the results indicates that the RSD for intralaboratory repeatability of some laboratories can be as high as 34% for a standard solution and 57% for the sediment sample, these results being balanced by the very good repeatability of the other laboratories. This variability using presumed identical methods and samples is high, and the parameters responsible of this variability should be identified.

The first round of interlaboratory comparison of dioxin-like compounds in food using bioassays (*24*) leads to quite similar conclusions: results are on the same order of magnitude when a similar or quite similar set of parameters is applied for the analysis of cod liver oil (1.8−26.9 pg TEQ/g) or fly ash extract (446−7361 pg TEQ/g), but the range of results is still broad and the RSD for reproducibility is high for some laboratories (up to 44%).

A better standardization of methods and an awareness of critical CALUX procedural characteristics that impact accuracy and precision of results are required.

**Can CALUX Be Used as a Tool for the Estimation of the "Global Dioxin-Like Toxicity of a Sample"?** The ideal measurement of the "global dioxin-like toxicity of a sample" due to all AhR ligands, including synergistic and antagonistic effects is impossible by CALUX, and probably by any analytical method, for the following reasons.

(1) CALUX bioassay is measuring an early biomarker of the response, a biological endpoint that is not directly responsible or apparently related to toxicity but is an effect that has been demonstrated to correlate with the toxic effect of dioxins and related compounds. In contrast, instrumental analysis approaches use TEFs, which are directly derived from toxicity studies and provide more accurate assessment of the toxic equivalency of a sample extract.

(2) A fraction of the AhR ligands in a given sample is discarded during the sample preparation. Thus, the overall response of the standard CALUX assay by a cleaned up sample does not fully measure all AhR ligands present in that sample.

(3) Some compounds, such as PAHs, give a positive response in CALUX, but these chemicals do not directly produce AhR dependent dioxin-like toxic effects. This is well-established in the literature (*26*, *27*, *29*, *30*) and is most likely due to the fact that these compounds are very rapidly metabolized and thus are not persistent enough to produce AhR-dependent adverse effects. Thus, inclusion of such a class of compounds in the TEQ estimation is not justified.

(4) The concentrations in TEQ are generally measured in CALUX using commercially available rodent cell lines and extrapolated to other species (fish, birds) for which the REP of the individual dioxin-like compounds can be extremely different (*28*).

Since "the global dioxin-like toxicity" cannot be estimated, the interpretation is easier when a selective cleanup is used

to isolate the compounds of interest. In this case, the CALUX response is related to the chemical concentrations of dioxin-like PCDDs/Fs and PCBs.

**Can the CALUX Bioassay Be a Replacement for GC–High-Resolution MS Analysis?** While CALUX bioassay-derived TEQs and GC–high-resolution MS-derived TEQs have been observed to be relatively close for numerous sample matrices (reviewed in Denison et al., *18*), CALUX and chemical analyses results can differ significantly and a variety of factors can contribute to this (*22*).

(1) CALUX analyzes the overall biological activity (i.e. gene induction ability) of all AhR ligands (agonists and antagonists) present in the particular extract, while chemical analyses focus on a selected numbers of compounds (i.e. those documented to produce AhR-dependent toxicity).

(2) CALUX bioassay REPs are species- and tissue-specific, and many of these REPs appear to be significantly different from the WHO-TEF (*28*) used in chemical analyses.

(3) In chemical analyses, nondetected compounds do not contribute to the total TEQ, or their contribution is estimated (commonly presented as one-half their detection limit), while in CALUX analyses, all AhR ligands (agonists and antagonists) present in the extract contribute to the final calculated bioassay TEQ.

(4) Nonadditive interactions (or at least a part) can be taken into account in CALUX analyses if all compounds are present in the cleaned up extract but not be determined in chemical analyses.

Considering these differences, results from CALUX and chemoanalyses can be very close for some samples but quite different for some other samples. These differences depend on the relative concentration of the different AhR ligands (agonists and/or antagonists), the particular CALUX cleanup method applied, the number of dioxin-like compounds analyzed, and detection limits of chemical analyses. Compared to instrumental methods of TEQ determination of sample extracts, the fact that the CALUX bioassay is not directly determining the toxic equivalency of a complex mixture but only its relative gene induction potency of TCDD equivalent means that it will not be an appropriate replacement for this method.

However, while CALUX and instrumental analysis are different tools, they are highly complementary and can provide comparable results with regard to the relative quantitation of dioxin-like HAHs. The predictiveness of the CALUX bioassay strongly supported its application as a method for screening and prioritization of samples for subsequent instrumental analysis.

**CALUX Applications.** Even given some of its limitations, the CALUX analysis is currently one of the best new tools for estimation of the relative TCDD equivalents (TEQ) of an unknown sample extract. Moreover, the method, combined with an appropriate cleanup procedure, provides a relatively cheap and rapid measurement of dioxin-like activity of an extract, even if the response does not necessarily reflect the actual "global dioxin-like toxicity". The equivalent measurement by chemical analysis would require the determination of the concentrations of hundreds of compounds per samples, meaning also that the TEF for each of these compounds needs to be determined. If only few compounds are analyzed, a toxic sample could be inaccurately considered as clean, since other major pollutants are simply not analyzed.

In most of the cases, it has been observed that CALUX will give an equal or higher response than that obtained/calculated from chemoanalyses, except if antagonists are present at relatively high concentration (and are not degraded or discarded during the cleanup). CALUX can thus be used as a valuable screening method for PCDD/F measurements in samples subjected to regulatory cutoff levels (*13*, *59*, *60*).

For environmental screening and analysis considerations, chemoanalysis and CALUX are complimentary. Chemoanalysis provides the concentration of specific compounds in the mixture, whereas the CALUX provides an overall biological response/potency of the mixture. A mass balance comparison between CALUX and chemoanalyses will suggest the magnitude of dioxin-like activity due to other compounds than those followed by chemoanalyses (*7*, *17*, *61*).

The rapidity and lower cost of CALUX analysis is attractive for the high number of samples required for environmental and epidemiological monitoring and for ensuring the lack of contamination of the food chain by dioxin-like chemicals. The key application role of CALUX bioassays is in screening and prioritization of samples.

In conclusion, it must be made clear that both CALUX and chemoanalysis results are only an approximation of the overall biological/toxicological potency of a sample. Indeed, the WHO-TEF values used in chemoanalysis are derived from several tests and indicate a 0.5–1 order of magnitude estimate of the toxic potency of a compound relative to TCDD (*62*). The concentrations measured are accurate, but the concentrations expressed in toxic equivalents remain an approximation. Further analysis and understanding of the AhR-dependent molecular mechanisms of toxicity of dioxin-like chemicals will improve our ability to assess and evaluate the toxic potential of complex mixtures of dioxin-like chemicals and to develop improved and more toxicologically relevant bioanalytical screening methods.

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## Supporting Information Available

Materials and methods for CALUX assays and PAH analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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