Environ. Sci. Technol. **2005**, *39*, 1741−1748

Validation and Interpretation of CALUX as a Tool for the Estimation of Dioxin-Like Activity in Marine Biological Matrixes

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Among the different analytical tools proposed as an alternative to the very expensive gas chromatography high-resolution mass spectrometry (GC-HRMS) analyses of polychlorodibenzo-*p*-dioxin and polychlorodibenzofurans, Chemically Activated LUciferase gene eXpression (CALUX) in vitro cell bioassay is very promising. It allows the analyses of a high number of samples since it is relatively fast, inexpensive, and sensitive. However, this technique is not yet widely applied for screening or environmental monitoring. The main reasons are probably the lack of validation and the difficulty in interpreting the global biological response of the bioassay. In this paper, the strict quality control criteria set up for the validation of CALUX are described. The validation has shown good repeatability (relative standard deviation (RSD) = 9%) and good within-lab reproducibility (RSD = 15%) of the results. The quantification limit, in the conditions applied in this paper, is 1.25 pg CALUX-TEQ/g fat. Comparison of CALUX and GC-HRMS analysis was made for various marine matrixes (fishes, mussels, starfishes, sea birds, and marine mammals). Good correlations are usually observed, but there are systematic differences between the results. Attempts are made to identify the origin of the discrepancy between the two methods.

Introduction

It is well established that dioxin and dioxin-like compounds produce a wide spectrum of toxic effects: carcinogenicity, teratogenicity, immunotoxicity, etc. (*1*, *2*). These compounds are now in the center of attention, especially after different incidents occurring during the past decades (Yusho, Seveso, Vietnam war, dioxin crisis in Belgium, contamination of citrus pulp, etc.). As a consequence, additional limits have been introduced in European legislations: emission limits for incinerators (directives 89/429/EEC, 89/369/EEC, 94/67/EC, 98/0289) and maximum levels in food and feed (*3*). Besides this, international conventions such as the OSPAR convention (Convention for the Protection of the Marine Environment of the North-East Atlantic) and the Protocol to the 1979 convention on long-range transboundary air pollution on persistent organic pollutants impose a reduction of the emission to background level and the identification and regular control of emission sources.

In that context, a rapid, inexpensive, and reliable method of analysis of dioxin and dioxin-like compounds is mandatory. Among the different analytical tools (*4*−*7*) proposed as an alternative to the very expensive gas chromatography high-resolution mass spectrometry (GC-HRMS) analyses of polychlorodibenzo-*p*-dioxin (PCDD) and polychlorodibenzofurans (PCDF), Chemically Activated LUciferase gene eXpression (CALUX) in vitro cell bioassay is very promising.

This bioassay is based on the fact that the dioxin toxicity is induced through the binding of dioxins and dioxin-like compounds to the aryl hydrocarbon receptor (AhR) (*8*−*10*). The complex is then translocated to the nucleus of the cell, where it induces the transcription of different genes (see Van Overmeire et al. (*11*) for a complete description). The cells used in the bioassay were genetically modified so that the complex induces the expression of the firefly luciferase gene and consequently the production of luciferase. In CALUX analyses, the cells are incubated with a solution containing dioxins and/or dioxin-like compounds. After incubation, the cells are lysed and luciferin is added. The reaction between luciferin and luciferase produces light that is measured with a luminometer. The measured luminescence is converted into a CALUX 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). One single global biological response is thus measured by CALUX for all AhR ligands present in the extract, including nonadditive interaction between compounds.

On the contrary, chemical analysis determines the concentration of each 2,3,7,8-chlorine-substituted PCDD/F. The concentrations are then multiplied by their specific toxic equivalent factor (TEF) to take the difference in toxicity of the different congeners into account (*12*, *13*). The concentrations expressed in the TEQ are then summed, assuming additivity of the responses.

The TEF value of a compound is determined by the comparison of different toxic or biological endpoints of this compound to those of the 2,3,7,8-TCDD, the most toxic congener, which is considered as reference. Until now, TEF values have been assigned only to 17 congeners of PCDD/F and 12 congeners of dioxin-like PCB (4 coplanar PCB (cPCB) and 8 mono-ortho PCB), but many other compounds are potential candidates (*12*). In CALUX, the relative potency (REP) value for one compound is the equivalent of the TEF value but is specific for one endpoint and one specie. The CALUX-REP values are usually consistent with the WHO-TEF values (Table 1) for PCDD/F but diverge more for dioxin-like PCB (*14*).

Since the principles of the CALUX and of GC-HRMS data are so different and since CALUX is a relatively new method, this technique is not yet widely applied for screening or environmental monitoring (*15*, *16*). The purpose of this paper deals with the validation of CALUX for the analysis of dioxin-like activity in diverse marine matrixes (fishes, mussels, starfishes, sea birds, and marine mammals). Repeatability, reproducibility, and quantification limits are evaluated, and the quality control criteria specifically designed for this

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10.1021/es049182d CCC: $30.25 © 2005 American Chemical Society
Published on Web 01/27/2005

TABLE 1. Comparison of TEF and CALUX REP Values[a] Obtained for the Mouse Cell Line PGudLuc 6.1

	human WHO-TEF (*12*)	CALUX REP (*14*)
Dioxins/Furans		
2,3,7,8-TCDD	1	1.00
1,2,3,7,8-PeCDD	1	0.73
1,2,3,4,7,8-HxCDD	0.1	0.075
1,2,3,6,7,8-HxCDD	0.1	0.098
1,2,3,7,8,9-HxCDD	0.1	0.061
1,2,3,4,6,7,8-HpCDD	0.01	0.031
OCDD	0.0001	0.0003
2,3,7,8-TCDF	0.1	0.067
1,2,3,7,8-PeCDF	0.05	0.14
2,3,4,7,8-PeCDF	0.5	0.58
1,2,3,4,7,8-HxCDF	0.1	0.13
1,2,3,6,7,8-HxCDF	0.1	0.14
1,2,3,7,8,9-HxCDF	0.1	0.11
2,3,4,6,7,8-HxCDF	0.1	0.31
1,2,3,4,6,7,8-HpCDF	0.01	0.024
1,2,3,4,7,8,9-HpCDF	0.01	0.044
OCDF	0.0001	0.0016
cPCB		
PCB 77	0.0005	0.0014
PCB 81	0.0001	0.0045
PCB 126	0.1	0.038
PCB 169	0.01	0.0011
Mono-Ortho PCB		
PCB 105[a]	0.0001	10^{-6}
PCB 114	0.0005	0.00014
PCB 118[a]	0.0001	10^{-6}
PCB 123[a]	0.0001	3×10^{-7}
PCB 156	0.0005	0.00014
PCB 157[a]	0.0005	3×10^{-6}
PCB 167[a]	0.00001	3×10^{-7}
PCB 189[a]	0.0001	2×10^{-7}

[a] REP values determined and communicated by XDS.

bioassay are presented. Attempts are made to explain the discrepancy between the two methods.

Materials and Methods

Cell Line. The pGudLuc 6.1 cell line was supplied by Xenobiotic Detection Systems, Inc (USA). This genetically modified cell line responds to dioxin-like chemicals with the induction of firefly luciferase in a time-, dose-, and AhR-dependent manner (*17*).

Reagents and Solvents. The hexane (pestanal, for residue analysis grade), the sulfuric acid (95−98%, ACS reagent), the toluene (for pesticide residue analysis), and the silica gel 60 for column chromatography were purchased from Fluka, Sigma-Aldrich (Germany). Acetone and ethyl acetate were for gas chromatography, suprasolv grade, and were purchased from Merck (Germany). Anhydrous sodium sulfate was ultra-resi analyzed grade and was obtained from Baker (The Netherlands). The standard solution of 2,3,7,8-TCDD (50 pg/μL) was purchased from AccuStandard Inc (USA).

Samples. All samples were collected in the North Sea, along the Belgian coast. The muscle of the flat fishes (*Limanda limanda* and *Solea solea*), the blubber of common porpoise (*Phoconena phocoena*), the liver of sea birds (common guillemot, *Uria aalge*), the pyloric caecas of starfishes (*Asteria rubens*), and the whole soft tissues of mussels (*Mytilus edulis*) were used for analysis. The cod liver oil used as quality control was a gift from the De Smet Group (Belgium) and was used as received. The concentrations measured by GC-HRMS for this sample are 5.4 pg TEQ/g fat for PCDD/F, 14.3 pgTEQ/g fat for cPCB, and 4.4 pgTEQ/g fat for mono-ortho PCB.

Extraction. Samples were lyophilized during 24−48 h depending on the sample size. Dry samples were extracted with hexane by pressurized liquid extraction (PLE) using a Dionex (USA) ASE 200 extractor. Thirty-three-mL cells were filled with freeze-dried samples and sodium sulfate. The extraction conditions were: 125 °C, 1500 PSI, 2 static cycles of 5 min. The extracts were dried upon sodium sulfate prior to concentration and gravimetric determination of fat. Part of the fat was analyzed by CALUX, and another part was analyzed by GC-HRMS.

GC-HRMS Analysis. Analyses were performed according to the procedure under accreditation described by Focant et al. (*18*). Purification was performed using the automated Power-Prep system from FMS (USA), and analysis was performed by GC-HRMS on a MAT95XL high-resolution mass spectrometer from Thermofinnigan (Germany) or on an Autospec Ultima from Micromass (U.K.), operating at a minimum of 10 000 resolution. TEQ concentrations were calculated using the WHO TEFs for humans (*12*).

CALUX Assay. A 25-mL Pyrex disposable column from Sigma Aldrich (Germany) was filled, from bottom to top, by 1.9 g of sodium sulfate, 2.8 g (for samples up to 0.25 g fat), 5.6 g (for samples up to 0.6 g fat), or 8.4 g (for samples up to 1. 5 g fat) of 33% (w/w) sulfuric acid silica gel and 1.9 g of sodium sulfate and rinsed with 30, 45, or 60 mL of hexane, according to the amount of sulfuric acid/silica gel. A 10-mL Pyrex disposable column from Sigma Aldrich (Germany) was filled from bottom to top by 0.7 g of sodium sulfate, 0.34 g of X-CARB, which is an activated carbon patented by Xenobiotic Detection Systems Inc (USA) and 0.7 g of sodium sulfate and rinsed with 5 mL of acetone, 20 mL of toluene and 10 mL of hexane. The acidic silica column was placed on top of the carbon column. The fat was weighed and dissolved in 5 mL of hexane. The extract was then loaded on the sulfuric acid silica gel column, and the vial was rinsed 2 times with 5 mL of hexane, which were added on the column too. The column was then eluted with 15, 30, or 45 mL of hexane, according to the size of the acidic silica column. When the elution was completed, the acidic silica column was removed and the carbon column was eluted with 8 mL of a hexane−acetone (90/10) mixture. This fraction was discarded. The fraction containing the cPCB (PCB fraction) was subsequently eluted with 15 mL of hexane/ethyl acetate/toluene (80/10/10), and the fraction containing PCDD/F (dioxin fraction) was eluted afterward with 20 mL of toluene. Extracts were concentrated to dryness in a centrifuge under vacuum and resuspended in a known volume of hexane. Prior to dosing the plate, the latter hexane solutions (1 mL) were transferred in 4 μL of DMSO using a centrifuge under vacuum, and finally, 400 μL of medium were added to each extract in DMSO.

Determination of the Percentage Recovery. Measurement programs for real samples include 13 unknowns, a procedural blank, a quality control sample, and one extra sample designed to determine the percentage recovery of the dioxin fraction. This extra sample was spiked with $^{14}C_{12}$-TCDD and purified according to the same procedure. Radioactivity measurements were performed on a Quantulus instrument from Perkin-Elmer (USA). The percentage recovery was calculated by dividing the radioactivity measurement of the dioxin fraction by the radioactivity measurement of the reference solution. The percentage recovery measured for this extra sample is used to correct the results of the unknowns of the same sample set. For the PCB fraction, the same percentage recovery of 80% was used to correct all the raw results, based on the percentage recovery measured for a standard solution of cPCB (chemical analysis); see below for more details.

Preparation of the Plate. Cells were cultured in phosphate-buffered saline medium with l-glutamine (Gibco, U.K.) and supplemented by 8% of fetal bovine serum provided by Hyclone Laboratories (U.S.A.), 45.5 unit/mL of penicillin and 45.5 unit/mL streptomycin provided by Gibco (U.K.), at 37

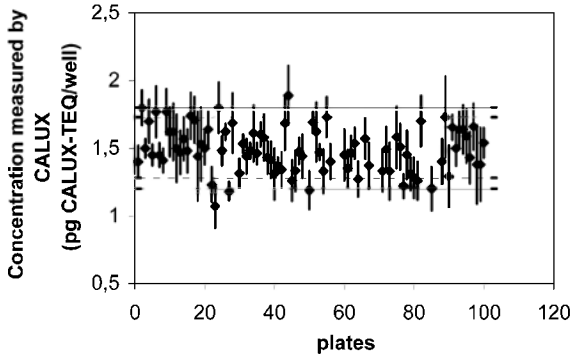

FIGURE 1. Control chart set up with the average (●) and standard deviation associated with 6 measurements by CALUX of the same standard solution of 2,3,7,8-TCDD on each plate. Dashed line, 1.5 pgCALUX-TEQ/well ± 15%, alarm limit; solid line, 1.5 pg CALUX-TEQ/well ± 20%, control limit.

°C and 5% CO_2 in an atmosphere saturated with water. For the CALUX bioassay, 96-well culture plates were seeded with 200 μL of cell suspension at a density of 85×10^4 cells/mL.

Dosing the Plate. On each plate, 10 DMSO solutions of TCDD (2 μL/well of solutions 25 000, 12 500, 6 250, 3 125, 1 562, 781, 391, 195, 98, and 49 fg TCDD/μL DMSO) were analyzed to draw the calibration curve. A solution of 750 fg TCDD/μL DMSO (prepared independently from calibration solutions) was analyzed in 6 wells, randomly distributed over the plate, for quality control (see below), and DMSO alone was analyzed in 4 wells. No analyses were performed in the external wells.

Each solution or extract is analyzed in 2 wells, and the mean of the results is used for calculation.

Reading the Plate. After 20−24 h of incubation, cells were examined microscopically for obvious toxicity. Each well was rinsed with 75 μL of PBS buffer, and afterward, 30 μL of cell culture lysis reagent from Promega (USA) were added to each well. The plate was shaken for 20 min at room temperature before being placed in the luminometer Lucy 1 from Anthos (Austria) for another 10 min. After addition of 50 μL of luciferase assay reagent from Promega (USA), the light output was integrated over 15 s after a delay time of 5 s, and results were expressed in relative light unit (RLU).

Analysis of Data. The average RLU value measured for DMSO alone was subtracted from all RLU values and an average RLU value for 2 wells with the same extract or solution was calculated. The best equation fitting the calibration curve was calculated using a four-variable Hill equation. This equation was used to convert the measured RLU value into data expressed in pg CALUX-TEQ/sample.

Results and Discussion

1. Quality Control Setting. Validation of this methodology requires strict quality control criteria, specifically designed for the CALUX bioassay and quite different from the ones used in chemical analyses.

The first quality control is designed to assess the quality of the plates. For that purpose, a standard TCDD solution (750 fg/μL DMSO) is prepared independently from the calibration solutions and analyzed in 6 dispersed wells of every plate (2 μL/well). Its concentration is chosen to perform the measurements in the lower linear part of the calibration curve, where measurements of real samples are also performed. Measurement averages as well as their standard deviations are plotted on control charts for which classical criteria are applied (Figure 1, which groups all results obtained by different operators, including students in formation, during 7 months using different standard solutions, different cell culture reagents, and different cells). Results must vary within the limits of the concentration of 1.50 pg CALUX-TEQ/well ± 20%. A second criterion imposes that the RSD associated with the 6 measurements does not exceed 15%. Both percentages of variation are based on previous results. If one of these criteria is not respected, results of the whole plate are rejected. On the basis of these criteria, 85% of the plates were accepted. The mean of data from accepted plates amounts to 1.50 pg CALUX-TEQ/well with RSD of 10%.

The second quality control is designed to check the absence of compounds, introduced by the procedure, which may change the response of the cells. For that purpose, the final extract of the procedural blank is spiked with the standard TCDD solution used for the quality control of the plates (750 fg/μL DMSO). The CALUX response of the latter mixture must be within the range, confined by the average − standard deviation (SD) and the average + SD, fixed for the standard solution. On one hand, responses in excess indicate contamination of the procedural blank with Ah ligands and/or the enhancement of the AhR-dependent gene expression by other methods. On the other hand, lower responses indicate the presence of compounds in the procedural blank, which decrease the cell's response. These compounds can lead to cell toxicity and death, inhibition of the AhR-dependent induction of gene expression (which can occur by many possible mechanisms), inhibition of luciferase enzyme activity, increased degradation of the luciferase, etc.

Third, a quality control sample is analyzed simultaneously with each series of samples, as is usually done within the framework of analytical method's accreditation. In this study, cod liver oil is used as quality control sample. A control chart is set up for which classical control criteria are applied. Results are discussed in detail in the next paragraphs.

Finally, when setting quantification limits, 3 factors were taken into account:

(1) The dose−response curves of some dioxin-like compounds do not necessarily reach the same maximum (efficacy) as the 2,3,7,8-TCDD dose−response curves, and this could lead to an underestimation of the contribution of these compounds to the CALUX-TEQ. When diluting the unknown samples so that the measurements are performed in the lower half of the calibration curve (concentration < 3125 fg/well), the inaccuracy due to the lower efficacy of the compounds should be decreased (*14*).

(2) The antagonistic effect of some compounds is lower when working at lower concentrations (within laboratory research, data not shown).

(3) The quantification at concentrations below our 7th point of calibration (781 fg/well) is less precise. This value is then considered as the "instrumental" quantification limit.

Consequently, it was decided to quantify only the samples giving raw results in the lower half of the calibration curve, limited by concentrations of 3125 fg/well and 781 fg/well. When needed, samples are diluted or concentrated to give raw results in that range.

Measurement programs for real samples include 13 unknowns, a procedural blank, a quality control sample, and an additional sample spiked with $^{14}C_{12}$ TCDD designed for the determination of the percentage recovery.

When correcting results with the percentage recovery, it is assumed that the behavior of the labeled TCDD is representative of all PCDD/F. This approximation can be easily accepted at least for the TCDD/F and PeCDD/F, which are responsible of the biggest part of the TEQ. The behavior of the more chlorinated PCDD/F may differ more, especially on the carbon column, but their contributions to the total TEQ are limited. It is also assumed that the percent recovery of all samples in one set of samples is the same. Again, it is an approximation, but as the percent recovery of the labeled TCDD did not vary a lot during one year (mean 78%, $n = 15$,

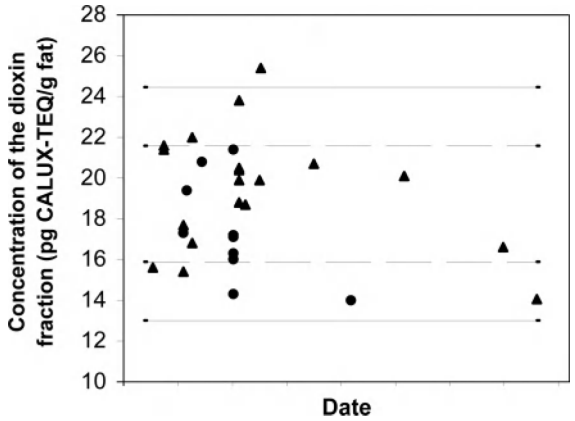

FIGURE 2. Control chart set up with CALUX results of the dioxin fraction of a quality control sample of cod liver oil. (●) 1 g fat; (▲) 500 mg fat; dashed line, mean ± SD; solid line, mean ± 2SD.

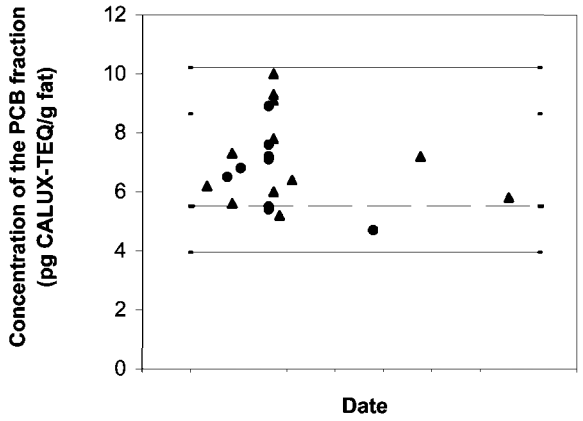

FIGURE 3. Control chart set up with CALUX results of the PCB fraction of a quality control sample of cod liver oil. (●) 1 g fat; (▲) 500 mg fat; dashed line, mean ± SD; solid line, mean ± 2SD.

RSD = 8%), a more precise determination of the recovery would bring more complication than advantage.

2. Validation for the Separated PCB and PCDD/F Fractions. During cleanup, the extract is split into a "dioxin fraction" and a "PCB fraction" on the carbon column placed after the acidic silica column. Specifications given by the carbon producer are as follows: (1) All coplanar PCB are in the PCB fraction, except the PCB 169, for which maximally 30% can be present in the dioxin fraction. (2) Since OCDD tends to bind strongly to the carbon, a minimum of 50% OCDD is collected in the dioxin fraction.

These separation properties were confirmed in our laboratory, using a standard solution of coplanar and mono-ortho PCB (chemical analysis). The percent recovery of the coplanar PCB 77, 81, 126, and 169 in the PCB fraction are 80, 84, 77, and 69% respectively. Only traces of these compounds are collected in the dioxin fraction (2−6%), except for the cPCB 169 (18%). About 30−40% of the mono-ortho PCB is collected in the PCB fraction, and only traces (<2%) are collected in the dioxin fraction (*19*).

By consideration that (1) only a small part of the mono-ortho PCB is collected in the PCB fraction and (2) the REP values of these mono-ortho PCB are very small, it is probable that the contribution of PCB mono-ortho to the concentration measured by CALUX in the PCB fraction is poor for most of the samples. CALUX results for the PCB fraction were then compared to the concentration of only the coplanar PCB, determined by GC-HRMS.

Dioxin Fraction. The repeatability, the intermediate precision, and the effect of the acidic silica column size on the results have been evaluated by 30 results of the cod liver oil used as quality control sample. The control chart with these data gathered during one year is shown in Figure 2.

As described in the Materials and Methods section, the amount of acidic silica gel used for cleanup is adjusted to the amount of fat to be analyzed. The size of the column has no statistically significant effect on the results (p = 0.78), therefore, all data are considered as one single data set in the next paragraphs.

The RSD for repeatability is 9% (n = 6), and the RSD for intermediate precision (within-lab reproducibility) is 15% (n = 30). Since the RSD associated with the measurement of the standard solution used to check the quality of the plate amounts to 10%, the sample preparation is highly reproducible.

The quantification limits of the method depend of the amount of fat that is used. For the maximum amount of fat used in this study (1.5 g), the quantification limit of the method is 1.25 pg CALUX-TEQ/g fat, taking into account that (1) the raw data has to be above 781 fg/well, (2) measurements are performed in 2 wells, (3) 80% of recovery is measured as a mean, (4) 1 of 1.2 mL are used to dose the plate since it is impossible to transfer all the extract without losses if there is no rinsing.

PCB Fraction. The RSD for intermediate precision of the PCB fraction (Figure 3), calculated for 20 samples of cod liver oil, is higher than the one for the dioxin fraction: 22% compared to 15%, respectively. Similarly, the RSD associated with repeatability is higher for the PCB fraction (19%, n = 6) compared to the dioxin fraction (9%). Nevertheless, the RSD associated with the measurements remains below 30%, as proposed by the European Commission (commission directives 2002/69/EC and 2002/70/EC) for bioassays, used as screening methods for the determination of dioxin in food and feedstuff.

The limit of quantification of the PCB fraction is the same than for the dioxin fraction since it is calculated in the same way.

More information is available when PCB and dioxins are analyzed separately in CALUX; the interpretation may be very different if only the sum of PCB and dioxins is considered. Results of the cod liver oil used as quality control illustrate it very clearly. On one hand, CALUX measures 18.7 pg CALUX-TEQ/g fat for the dioxin fraction, which is about 3 times more than the 5.4 pg TEQ/g fat measured by GC-HRMS for the PCDD/F. On the other hand, CALUX measures 7.1 pg CALUX-TEQ/g fat for the PCB fraction, which is about 2.5 times less than the 18.7 pg TEQ/g fat measured by GC-HRMS for cPCB and mono-ortho PCB. However, if we consider only the total TEQ obtained by CALUX and GC-HRMS, the results are very close: 25.8 pg CALUX-TEQ/g fat and 24.1 pg TEQ/g fat, respectively.

3. Application of the CALUX Methodology to Marine Biological Matrixes and Comparison with Chemical Analysis. The trueness of CALUX is much more difficult to evaluate. Chemical analyses of all dioxin-like compounds would be impossible, and TEF values are not available for every Ah ligands. Moreover, nonadditive interactions between compounds would not be taken into account. Traditionally, results of the chemical analyses of PCDD/F and dioxin-like PCB are used as the reference. In this study, CALUX results for the dioxin fraction and GC-HRMS results for the PCDD/F are compared for the muscle of flat fishes (*Limanda limanda* and *Solea solea*), the blubber of marine mammals (Common porpoises, *Phoconena phocoena*), the liver of sea birds (common guillemot, *Uria aalge*), the pyloric caecas of starfishes (*Asteria rubens*), and the whole soft tissue of mussels (*Mytilus edulis*), collected along the Belgian coast. CALUX results for the PCB fraction and GC-HRMS results for cPCB are compared for the same samples of sea birds and mussels

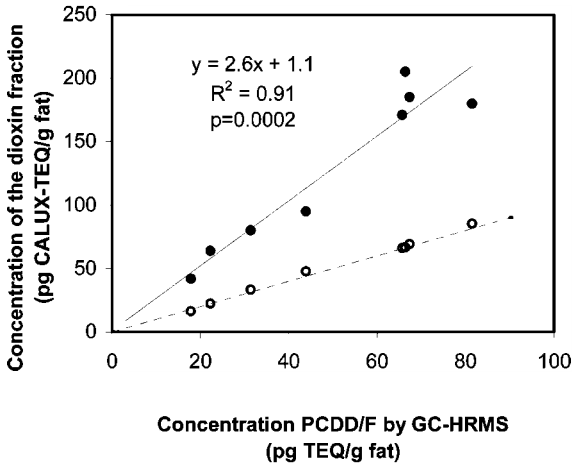

FIGURE 4. Comparison of CALUX results for the dioxin fraction and concentrations of PCDD/F by GC-HRMS for flat fish's muscle samples (lower-bound concentrations). (●) Dioxin fraction in CALUX; (○) PCDD/F by GC-HRMS recalculated using REP values; dashed line, $x = y$.

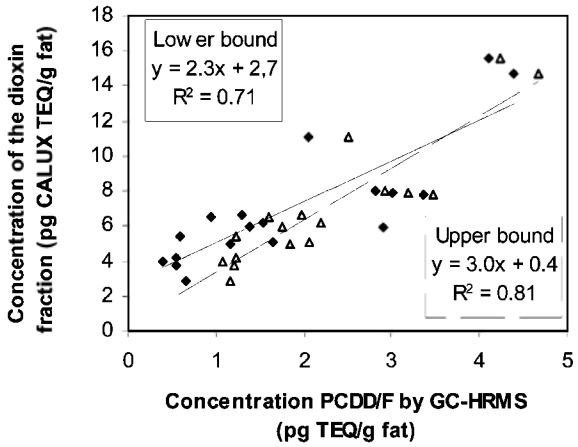

FIGURE 5. Comparison of CALUX results for the dioxin fraction and concentrations of PCDD/F by GC-HRMS for marine mammal's blubber samples. (●) Lower-bound values in GC-HRMS; (△) upper-bound values in GC-HRMS.

(PCB results are not available for starfishes, marine mammals, and fishes in CALUX and/or in GC-HRMS).

To limit the number of parameters to be compared, extraction was performed in one lab, and the fat extracted was homogenized and shared between the laboratories for CALUX and GC-HRMS analysis. Since the fat level is very low in some matrixes, the concentrations expressed on a fat base are quite high for those matrixes.

Dioxin Fractions. Fish. Eight samples of flat fish's muscles (6 samples of *Limanda limanda* and 2 samples of *Solea solea*) were analyzed, and the correlation between the results of the dioxin fraction obtained by the CALUX method and the PCDD/F TEQ value obtained by GC-HRMS are shown in Figure 4.

Statistical parameters show that the results are highly correlated, with an R^2 value of 0.91 and $p \ll 0.001$.

The average CALUX/GC-HRMS ratio amounts to 2.6, meaning that CALUX results are approximately 2.6 times higher than GC-HRMS results. Different factors can explain the observed bias:

(1) the difference between TEF and REP values; (2) antagonistic or synergistic effects; (3) the quantification limits and the presence of interferences in GC−HRMS analysis; and (4) the presence of other AhR ligands.

To investigate the relative importance of the different parameters, the concentrations measured by GC-HRMS were multiplied by the corresponding REP values instead of the TEF values (Table 1). These values are represented by open circles in Figures 4, 7, 8, and 9. The values calculated in this way are very close to the values obtained by using the TEF values, as represented by the dotted line with a slope of 1 in Figures 4, 7, 8, and 9. The difference between the TEF and REP values is, therefore, not responsible of the observed discrepancy between the two methods for all matrixes investigated.

Synergistic effects are not common in CALUX. Corticosteroids, e.g., induce a very weak response in CALUX but dramatically enhance the response of TCDD (*20*). However, they are degraded during the cleanup, probably together with all compounds of this class, which are susceptible to enhance the CALUX response. Antagonistic effects are much more common and are described for PCB (see Safe (*21*) for a review), α-naphthoflavone (*22*), polychloronaphthalenes, and hexachlorobenzene (*22*, *23*). During the cleanup, PCB, most of the polyaromatic hydrocarbons (PAH), and hexachlorobenzene are discarded from the dioxin fraction (*23*), but some other antagonistic compounds may still be present. The result of the dioxin fraction can thus be underestimated compared to results for which no interaction occurs between compounds.

When some congeners, representing an important part of the TEQ value, are not detected in GC-HRMS, or when there is an interference for one of these congeners, the concentrations measured by GC-HRMS can be underestimated. In CALUX analyses, this bias is not observed since one single overall response, expressed in pg CALUX-TEQ/g, is measured. Therefore, the discrepancy between the two methods can be due, in some cases, to the limit of quantification or interferences in GC-HRMS analysis.

For all samples except blubber of common porpoise, most of the congeners were detected by GC-HRMS. The higher values for the dioxin fractions measured by CALUX, compared to the PCDD/F concentrations measured by GC-HRMS are then probably due to the presence of other AhR ligands. The list of identified AhR ligands is already very long, as evidenced by Behnisch et al. (*5*). These authors list REP values of some AhR agonists for different bioanalyses and provide a diagnosis of the pollutants' sources, fates, and levels in the environment. Additional information is available in papers from Murk et al. (*24*), Denison et al. (*8*, *25*−*27*), Hoogenboom et al. (*20*), and Seidel et al. (*28*).

At first sight, it may be surprising that CALUX and GC-HRMS results are so well correlated since the concept of analysis is completely different for the 2 methods. However, GC-HRMS analyzes only the PCDD/F, whereas CALUX analyzes PCDD/F plus other AhR ligands. Consequently, a good correlation between CALUX and GC-HRMS can be observed when the ratio of PCDD/F to other AhR ligands is constant.

Further research is needed to identify these "other AhR ligands" but also to evaluate their potential dioxin-like toxicity. Indeed, a positive response in CALUX implies activation of the AhR-dependent gene expression by the compound but does not take into account the other toxic or biological endpoints, in particular metabolism and accumulation, which are important parts of the toxicity associated with dioxins (*12*).

Marine Mammals. The correlation between the results of the dioxin fractions measured with CALUX and the PCDD/F TEQ values obtained by GC-HRMS for eighteen samples of common porpoise (*Phoconena phocoena*) blubber is illustrated in Figure 5.

At these low to very low levels, some and sometimes most of the congeners of PCDD/F are not detected in GC-HRMS. The concentrations measured by GC-HRMS are, therefore,

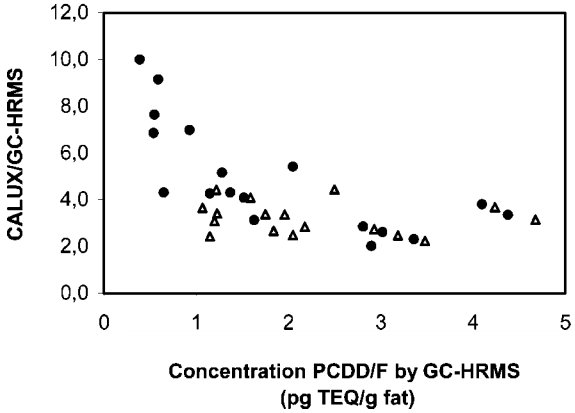

FIGURE 6. Variation of the ratio CALUX/GC-HRMS for marine mammals samples when lower- or upper-bound values are used in GC-HRMS. (●) Lower-bound values in GC-HRMS; (△) upper-bound values in GC-HRMS.

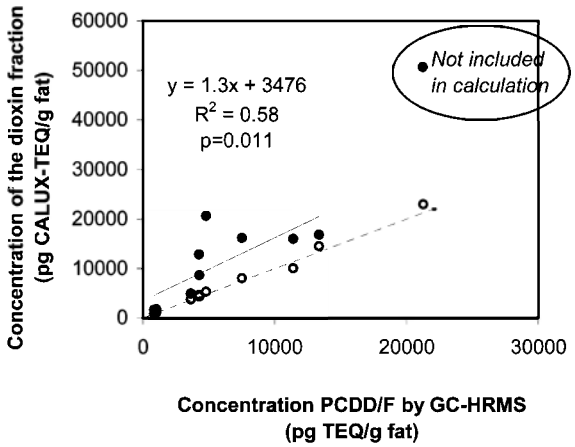

FIGURE 7. Comparison of CALUX results for the dioxin fraction and concentrations of PCDD/F by GC-HRMS for sea bird's liver samples (lower-bound concentrations). (●) Dioxin fraction in CALUX; (○) PCDD/F by GC-HRMS recalculated using REP values; dashed line, $x = y$.

underestimated when the lower-bound values (the concentration of nondetected compounds is set equal to 0) are used. In CALUX analysis, this measurement bias is not observed. As a consequence, the ratio between CALUX and GC-HRMS increases to 10 at the lower concentrations, when the number of nondetected congeners is important (Figure 6).

When the upper-bound values (the concentration of nondetected compounds is set equal to the quantification limit) are used instead of the lower-bound values for GC-HRMS results, the correlation between CALUX and GC-HRMS is better (Figure 5), and the CALUX to GC-HRMS ratios are more constant and vary between 2 and 4 (Figure 6).

Sea Birds. The concentration of one sample was much higher than the 10 other ones. This sample was not included in the statistic calculation since it raises the correlation coefficient to 0.82, which is not representative of the correlation for the other samples. Indeed, for the other samples, CALUX and GC-HRMS results are still correlated but less than for the other matrixes ($R^2 = 0.58$ and $p = 0.011$) (Figure 7).

In some cases, the CALUX measurement is very close to the response expected if only PCDD/F contribute to the response (PCDD/F concentrations determined by GC-HRMS multiplied by the REP values). In some other cases, PCDD/F represent only one-half to one-third of the CALUX response, as it was observed for fishes and marine mammals. The ratios between PCDD/F and other AhR ligands are thus less constant

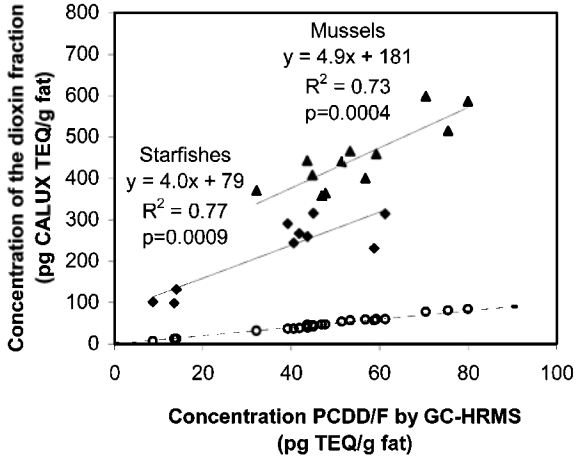

FIGURE 8. Comparison of CALUX results for the dioxin fraction and concentrations of PCDD/F by GC-HRMS (lower-bound concentrations) for mussels (whole soft tissues) and starfishes (pyloric caecas) samples. (◆) Dioxin fraction in CALUX for starfishes; (▲) dioxin fraction in CALUX for mussels; (○) PCDD/F by GC-HRMS recalculated using REP values; dashed line, $x = y$.

than those observed for the other matrixes. This larger range of the ratio may be linked to environmental parameters such as different origin and available food.

Mussels and Starfishes. Results of the dioxin fraction measured by CALUX and PCDD/F TEQ values measured by GC-HRMS are well correlated for mussels ($n = 12$) and starfishes ($n = 10$) (Figure 8), with R^2 values of, respectively, 0.73 and 0.77 and $p \ll 0.001$.

However, the ratio of CALUX results to chemical results varies between 4 and 12 with an average of 8 (SD = 1.9), which implies that PCDD/F represent, as a mean, only one eight of the CALUX response. This contrasts with the results obtained for sea birds, marine mammals, and fishes for which PCDD/F represent, as a mean, one-third of the response (average ratio of CALUX results to chemical results of 2.8 (SD = 1.1, range 1.3−5.4)).

One possible explanation for this difference between vertebrates and invertebrates may be linked to the difference in the Ah receptor; an AhR homologue has been identified in several invertebrates but is unable to bind typical AhR ligands (*29*). Since biotransformation systems of invertebrates are different from those of vertebrates, it would not be surprising that the ratio between PCDD/F and other classical AhR ligands is different in vertebrates or invertebrates. For example, PAH is known to bioaccumulate in mussels (*30*) and to be quickly metabolized in vertebrates (*25*, *31*−*33*).

PCB Fraction. Sea birds. For the 10 results available for sea birds, the concentrations measured in the PCB fraction in CALUX are highly correlated to the cPCB concentrations measured by GC-HRMS (Figure 9). However, and in contrast with results obtained for dioxins, CALUX results are always lower than GC-HRMS results. When the cPCB concentrations measured by GC-HRMS are multiplied by the REP value instead of the TEF value (values represented by open circles in the Figure), the values calculated are usually close to the results obtained in CALUX. This means that the difference between TEF and REP explains most of the discrepancy between CALUX and chemical analysis.

In chemical analysis, the coplanar PCB 126 is usually responsible for more than 90% of the PCB TEQ. As the REP for the PCB 126 is roughly one-third of the TEF, the concentrations measured in CALUX is roughly one-third of the concentration measured by GC-HRMS.

Mussels. Since the range of cPCB concentrations measured in mussels is narrow and considering the quite high relative standard deviation associated with the measurements in

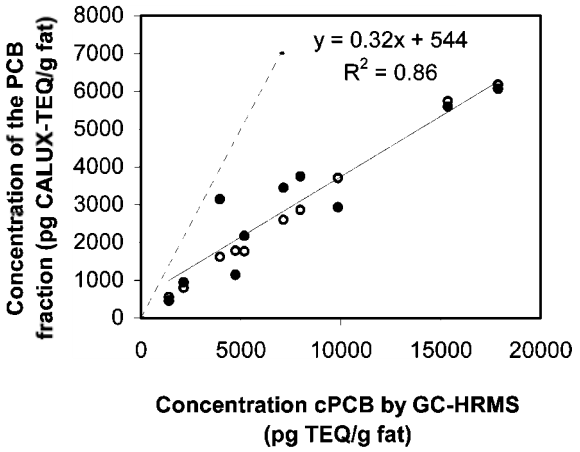

FIGURE 9. Comparison of CALUX results for the PCB fraction and concentrations of cPCB by GC-HRMS for sea birds samples. (●) PCB fraction in CALUX; (○) cPCB by GC-HRMS recalculated using REP values; dashed line, $x = y$.

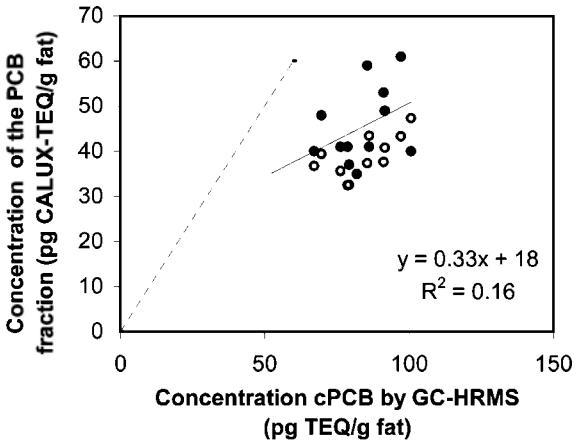

FIGURE 10. Comparison of CALUX results for the PCB fraction and concentrations of cPCB by GC-HRMS for mussels samples. (●) PCB fraction in CALUX; (○) cPCB by GC-HRMS recalculated using REP values; dashed line, $x = y$.

CALUX (22%), no correlation is found between concentrations of cPCB measured by GC-HRMS and CALUX for mussels (Figure 10). Nevertheless, the concentrations measured in CALUX are roughly one-third of the concentration measured by GC-HRMS.

As a whole, good correlations are observed between the dioxin fractions measured in CALUX and GC-HRMS analyses of PCDD/F for all matrixes investigated and those for very low (pg CALUX-TEQ/g fat) to very high concentrations (10 ng CALUX-TEQ/g fat). For fishes, sea birds, and marine mammals, CALUX measures approximately 2.8 times more than GC-HRMS. The difference is probably due to the presence of other AhR ligands. For mussels and starfishes (invertebrates), CALUX measures approximately 8 times more than GC-HRMS. The good correlations observed imply that the ratio PCDD-F/other AhR ligands is quite constant.

Good correlation is observed between the PCB fraction measured in CALUX and GC-HRMS analysis of cPCB for sea birds, but, in this case, CALUX measures approximately 3 times less than chemical analysis. The discrepancy between the 2 methods is mainly due to the difference between TEF and REP, especially for the PCB 126, which is responsible of more than 90% of the cPCB TEQ in these samples.

At low concentration, when some congeners that represent an important part of the TEQ value are not detected in GC-HRMS or when there is an interference for one of these congeners, the concentrations measured by GC-HRMS can be underestimated. In CALUX analysis, this bias of measurement is not observed since one global response, expressed in pg CALUX-TEQ/g, is measured for all AhR ligands. A better correlation between the 2 methods is observed when the upper-bound values (when available) are used for GC-HRMS results, instead of the lower-bound values.

CALUX and GC-HRMS are complementary. On one hand, CALUX is relatively inexpensive, rapid, quantitative, reproducible, and sensitive. Moreover, CALUX is able to detect the presence of AhR ligands other than PCDD/F and dioxin-like PCB. Its application to the numerous samples needed for environmental monitoring or environmental research would be easier than GC−HRMS determination of PCDD/F and dioxin-like PCB. The antagonistic effects that can affect the results are, however, very difficult to estimate. On the other hand, chemical analyses provide precise concentration and additional information about the contamination pattern.

Under the strict quality control especially designed, the CALUX method described in this paper has proven to be a valuable tool for the estimation of the dioxin-like activity in biological marine matrixes.

Acknowledgments

This study was financed by the "Impulse Program in Marine Sciences supported by the Belgian state, The Prime Minister's Services, Scientific, Technical and Cultural Affairs" (MN/12/95). The authors thank Jeff Sturkey for English grammar correction.

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Received for review June 2, 2004. Revised manuscript received December 1, 2004. Accepted December 6, 2004.

ES049182D