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INTRA- AND INTERLABORATORY CALIBRATION OF THE DR CALUX® BIOASSAY FOR THE ANALYSIS OF DIOXINS AND DIOXIN-LIKE CHEMICALS IN SEDIMENTS

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Abstract—In the Fourth National Policy Document on Water Management in the Netherlands [1], it is defined that in 2003, in addition to the assessment of chemical substances, special guidelines for the assessment of dredged material should be recorded. The assessment of dredged material is based on integrated chemical and biological effect measurements. Among others, the DR CALUX® (dioxin responsive–chemically activated luciferase expression) bioassay has tentatively been recommended for inclusion in the dredged material assessment. To ensure the reliability of this bioassay, an intra- and interlaboratory validation study, or ring test, was performed, organized by the Dutch National Institute for Coastal and Marine Management (RIKZ) in cooperation with BioDetection Systems BV (BDS). The intralaboratory repeatability and reproducibility and the limit of detection (LOD) and quantification (LOQ) of the DR CALUX bioassay were determined by analyzing sediment extracts and dimethyl sulfoxide (DMSO) blanks. The highest observed repeatability was found to be 24.1%, whereas the highest observed reproducibility was calculated to be 19.9%. Based on the obtained results, the LOD and LOQ to be applied for the bioassay are 0.3 and 1.0 pM, respectively. The interlaboratory calibration study was divided into three phases, starting with analyzing pure chemicals. During the second phase, sediment extracts were analyzed, whereas in the third phase, whole sediments had to be extracted, cleaned, and analyzed. The average interlaboratory repeatability increased from 14.6% for the analysis of pure compound to 26.1% for the analysis of whole matrix. A similar increase in reproducibility with increasing complexity of handlings was observed with the interlaboratory reproducibility of 6.5% for pure compound and 27.9% for whole matrix. The results of this study are intended as a starting point for implementing the integrated chemical–biological assessment strategy and for systematic monitoring of dredged materials and related materials in the coming years.

Keywords—CALUX® Dredged materials Dioxins Interlaboratory calibration Screening bioassay

INTRODUCTION

It is generally accepted that marine sediments form a sink for hydrophobic pollutants such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs). Since these classes of compounds tend to bioaccumulate in aquatic organisms inhabiting such polluted areas, they may pose a health risk for aquatic wildlife and also for humans through the consumption of contaminated fish. The occurrence of certain diseases in fish populations has been related to environmental pollutants. Pollution of the aquatic environment has been assumed to contribute (at least in part) to the etiology of fish diseases such as skin and liver tumors and fin rot [2–4]. The main source of contamination in the North Sea is through riverine outflows, primarily from the Rhine, Scheldt, and Meuse (The Netherlands), Elbe and Weser (Germany), and Thames (United Kingdom). As a consequence, coastal areas and estuaries are significantly polluted by PCDDs, PCBs, and other persistent organic pollutants [5,6]. In order to monitor the extent of contamination, the Dutch government has stated that the assessment of dredged materials will be based on integrated chemical and biological effect measurements [1,7]. The DR CALUX® (dioxin responsive–chemically activated luciferase expression) bioassay has been recommended for inclusion in the dredged material assessment for the analysis of dioxins and/or dioxin-like chemicals.

Traditional techniques for the detection and quantitation of PCDDs, PCDFs, and PCBs in sediments involve costly and time-consuming instrumental methods, such as high-resolution gas chromatography separation and mass spectrometry (HRGC/MS), making extensive monitoring of sediments difficult. Although these techniques provide information on the presence and concentration of individual congeners, no direct information is provided on the total biological (toxic) activity of such compounds in complex mixtures. The major advantage of using mechanistic-based bioassays for the assessment of dredged materials, such as the DR CALUX bioassay, is that instead of analyzing specific individual congeners, it determines the total biological (toxic) activity of groups of chem-

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icals with a similar toxic mode of action [7]. However, traditional analytical chemical techniques such as HRGC/MS are essential to determine the exact nature of individual congeners present in the samples under investigation.

The DR CALUX bioassay comprises a genetically modified H4IIE rat hepatoma cell line, incorporating the firefly luciferase gene coupled to dioxin responsive elements (DREs) as a reporter gene for the presence of dioxins and/or dioxin-like compounds [8–11]. In the DR CALUX bioassay, the induction of luciferase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is dose dependent. Hence, a 2,3,7,8-TCDD calibration curve can be used to quantify the total dioxin and/or dioxin-like content of a sample under investigation. Whereas concentrations of individual polyhalogenated aromatic hydrocarbons (PHAHs) as determined using HRGCMS have to be multiplied by their respective toxic equivalent factor (TEF) value and added up to give the total 2,3,7,8-TCDD toxic equivalent (TEQ) [12–14], the DR CALUX bioassay directly measures the arylhydrocarbon receptor (AhR)–related toxic potency of a mixture. The DR CALUX bioassay has been successfully used for the analysis of dioxins and/or dioxin-like chemicals in a wide variety of matrices, such as (human) serum, (human) milk, fish, fish oil, citrus pulp, and so on [15–20]. In addition, a number of papers have been published describing the validation of the bioassay and describing the correlation between DR CALUX and HRGCMS derived 2,3,7,8-TCDD TEQs [21–24].

To ensure the reliability of the DR CALUX bioassay for monitoring dredged materials, the accuracy and repeatability of the DR CALUX bioassay has to be determined. Therefore, both intra- and interlaboratory calibration studies were performed. Six laboratories, located in The Netherlands, the United Kingdom, Japan, and Belgium, were selected to participate. Each of these laboratories analyzed anonymous sediment samples in each of the three phases of the study. The participants were asked to perform the analyses according to supplied protocols. In addition, they were asked to extract and clean up sediments according to the procedure developed and validated by The Netherlands Institute for Fisheries Research (RIVO), in cooperation with the Dutch National Institute for Coastal and Marine Management. The protocol for the analysis of dioxin and/or dioxin-like content in sediment extracts (DR CALUX bioassay) was recently modified by BioDetection Systems BV (BDS) (SOP Dutch National Institute for Coastal Marine Management SPECIE*07). OpdenKamp performed the statistical analyses necessary to interpret the results of this interlaboratory validation study. The results of these studies are presented here and are intended as a starting point for implementation of the DR CALUX bioassay in the assessment of dredged materials for systematic monitoring in the coming years.

MATERIALS AND METHODS

Chemicals

The 2,3,7,8-TCDD was purchased from LGC Promochem (Wesel, Germany). The PCB 126 and PCB 169 were obtained from Labor Dr. Ehrenstorfer (Augsburg, Germany). Ultraclean DMSO and ethylenediaminetetraacetic acid (EDTA) were obtained from Acros (New Brunswick, NJ, USA). Ultra resi-analyzed *n*-hexane, sodium sulfate, and cleaned and ignited sea sand were from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Silica 60 (63–200 μm) was obtained from Merck (Darmstadt, Germany). Sulfuric acid (95–97%) was from Riedel de Haën (Seelze, Germany). Minimal essential medium (α-MEM with phenol red as pH indicator), fetal calf serum (Australian origin), and trypsin were purchased from Gibco, Invitrogen (Breda, The Netherlands). Dithiothreitol (DTT) and Luciferin was from Duchefa Biochemie B.V. (Haarlem, The Netherlands). Coenzyme A (free acid grade I) was from Roche Diagnostics (Mannheim, Germany).

Intralaboratory study

The intralaboratory calibration study was performed by the Institute for Environmental Studies. Sediment was extracted and cleaned up as indicated here. The determination of dioxin and/or dioxin-like content was according to the method indicated under the section DR CALUX analysis. For the intralaboratory study, the following parameters were investigated: limit of detection (LOD), limit of quantitation (LOQ), and reproducibility and repeatability of the bioassay.

Interlaboratory study

Project description. Six laboratories, located in The Netherlands, the United Kingdom, Japan, and Belgium, were selected to participate. Each of these laboratories analyzed blind samples in each of three phases of the study. The participants were asked to perform the analyses according to supplied protocols (see the following discussion). All samples supplied were analyzed three times. In addition, each single sample was analyzed in triplicate. All the laboratories participating in the interlaboratory study for the validation of the DR CALUX bioassay for dioxins and dioxin-like chemicals in sediment had experience in running the DR CALUX bioassay. However, none of the participating laboratories had prior experience with the extraction protocol to be used. Furthermore, the participants were free to use the dilution factor of their choice unless indicated otherwise.

Phase 1. The first phase of the interlaboratory study consisted of the DR CALUX analysis of two defined standard solutions (2,3,7,8-TCDD in DMSO; TCDD/PCB-126/PCB-169 mix in DMSO). The standard solutions were prepared by the Institute for Environmental Studies, Vrije Universiteit Amsterdam, The Netherlands, and sent to the participants. In addition, the participants received a complete concentration range of 2,3,7,8-TCDD in DMSO to be used as a TCDD calibration curve (a total of eight different TCDD concentrations, 0–300 pM 2,3,7,8-TCDD/well, including DMSO as a blank control). This calibration curve was used throughout the whole interlaboratory study. Furthermore, each participant analyzed its own TCDD calibration curve. Participating laboratories received three vials containing a TCDD stock solution in DMSO and three vials containing a TCDD/PCB-126/PCB-169 mix in DMSO. Dilutions of the stock solutions were prepared by the participants in DMSO and tested for dioxin and/or dioxin-like content. Raw data as well as converted data were used for statistical evaluation.

Phase 2. In the second phase of the study, the participants were asked to analyze three extracted and cleaned sediment samples using the DR CALUX bioassay. Sediments used for extraction and cleanup were freshwater sediments from the Western Scheldt, The Netherlands. The sediment extracts were prepared by the Royal Institute for Fishery Research (RIVO-DLO), IJmuiden, The Netherlands, according to the protocol given here. Dilutions of the supplied sediment extracts were prepared by the participants in DMSO and tested for dioxin and/or dioxin-like content. On each 96-well microtiter plate,

a 2,3,7,8-TCDD standard calibration curve was analyzed. Raw data as well as converted data were used for statistical evaluation.

Phase 3. During phase 3 of the interlaboratory study, participants received an identical sediment sample (freshwater sediment from the Western Scheldt, The Netherlands). The participants were asked to extract and clean up the sediment in three separate sessions according to the supplied protocol. Following extraction and cleanup, the three sediment extracts were analyzed in the DR CALUX bioassay. Participants were not instructed on the dilution to be used. Dilutions of the sediment extracts were prepared by the participants in DMSO and tested for dioxin and/or dioxin-like content. On each 96-well microtiter plate, a 2,3,7,8-TCDD standard calibration curve was analyzed. Furthermore, an appropriate commercially available procedure blank (washed and ignited sea sand; Baker, catalog 0252) was extracted, cleaned, and analyzed using the exact same protocols. Raw data as well as converted data were used for statistical evaluation.

Preparation of samples for the intra- and interlaboratory studies

Defined standard solutions. The two defined standard solutions were prepared by dissolving either 2,3,7,8-TCDD or 2,3,7,8-TCDD, PCB 126, and PCB 169 in DMSO. The 2,3,7,8-TCDD standard solution contained 2,3,7,8-TCDD in DMSO at a concentration of 7.5 nM. Sample 2 contained a mixture of 2,3,7,8 TCDD, PCB 126, and PCB 169 at concentrations of 5.0, 25, and 250 nM, respectively. The total 2,3,7,8-TCDD TEQ content of this mixture was calculated using both World Health Organization (Paris, France) WHO-TEF values and DR CALUX-relative potency (REP) values [25] and found to be 10 and 7.5 nM 2,3,7,8-TCDD TEQ, respectively. Overall, 27 individual measurements per sample and per participant were available for data analysis.

Extraction of sediment samples. Prior to extraction, sediment samples were freeze-dried and homogenized. Approximately 10 g of dried sediment were placed in a preextracted thimble, and a small piece of silanized glass wool was placed in the thimble on top of the sample to prevent sediment parts from leaving the thimble. The thimble was placed in a Soxhlet setup and extract for 16 h (overnight) with 200 ml hexane/acetone (3/1 v/v). The extracts were concentrated in the rotation evaporator until approximately 5 ml (p = 0.05 bar; T = 40°C) of extract remained. If the extract still contained solid particles, the extract was filtered with diatomaceous earth or sodium sulfate. The extract was transferred to a diatomaceous earth– or sodium sulfate–filled funnel and flushed with 10 ml hexane. The eluted extract was evaporated again in the rotary evaporator until approximately 5 ml of extract remained. The extract was transferred to a cleaned glass tube and concentrated until near dryness. The dried extract was finally redissolved in 3 ml hexane.

Cleanup of sediment samples. The extracted sediment samples were cleaned up using a multilayer column. The multilayer glass column consisted of the following materials (from top to bottom): 1 cm water-free sodium sulfate, 1 g silica, 7 g 44% sulfuric acid on silica, 1 g silica, 2 g 33% sodium hydroxide on silica, 1 g silica, 1.5 g 10% silver nitrate on silica, and a small piece of silanized glass wool. After addition of each layer, the column was compacted by tapping the column. After moistening and preelution with 25 ml of hexane of the column, the complete extract was transferred on the top of the column. The column was eluted with 130 ml of hexane, after which the eluate was concentrated on the rotation evaporator until approximately 5 ml remained (p = 0.2 bar; T = 40°C). The concentrated cleaned sediment extract was transferred to a clean glass tube and further concentrated to near dryness under a gentle stream of nitrogen. The extract was redissolved in 50 μl DMSO.

DR CALUX analysis

The DR CALUX cells were cultivated in minimal essential medium (α-MEM) supplemented with 10% fetal calf serum under standardized conditions (37°C, 5% CO_2, 100% humidity). The DR CALUX analyses of samples in DMSO of the three phases were performed in 96-well cell culture plates (Greiner). Cells were seeded in 100 μl growth medium and incubated for 24 h under standardized conditions until the cells reached a confluence of at least 95%. An additional 100 μl of growth medium were added to the wells containing the samples in DMSO. The final DMSO concentration in the wells was 0.4%. After 24 h of incubation, the exposure medium was removed, and the cells were rinsed with diluted phosphate buffered saline (demi water/[PBS]; 1/1, v/v). Thirty microliters of lysis mix (25 mM Tris, 2 mM dithiothreitol [DTT], 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*-tetra-acetic acid [CDTA], 10% glycerol, and 1% Triton x-100 [Sigma, St. Louis, MO, USA] pH 7.8) were added to each well and incubated at 4°C for at least 30 min, after which the microtiter plates were frozen at −80°C for a minimum of 30 min and a maximum of 1 d to lyse the cells.

The luciferase activity was measured using a luminometer equipped with two dispensers. The microtiter plates were thawed and shaken for 2 min at room temperature and placed in the luminometer. One hundred microliters of glow mix (20 mM trycin, 1.07 mM magnesium hydroxide carbonate pentahydrate, 2.67 mM magnesium sulfate, 0.1 mM ethylenediaminetetraacetic acid, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin) were automatically injected into each well. The light output was recorded on which the reaction was stopped by automatic injection of 100 μl of 0.2-M NaOH.

On each 96-well microtiter plate, a complete 2,3,7,8-TCDD standard concentration range was incubated and analyzed in triplicate. A curve fit of the 2,3,7,8-TCDD standard range was produced for the calculation of DR CALUX TEQ content in the samples tested. The analyzed relative light units (RLU) from the samples were interpolated on the 2,3,7,8-TCDD standard curve, and the DR CALUX TEQ content was quantified between the limit of quantitation (LOQ) and the concentration of 2,3,7,8-TCDD at which 50% of the maximum response is observed (EC50).

Statistical analysis

To maintain consistency of statistical analyses, an identical microtiter plate setup was used by all participants, and all samples were analyzed in an identical manner. Both raw data and pretreated data from analyzed samples were submitted to OpdenKamp Registration and Notification for statistical evaluation. Data pretreatment consisted of all necessary calculations to convert the luminosity readings as submitted by the participating laboratories to effective dioxin-receptor activity (pM 2,3,7,8-TCDD TEQ). In addition to the analysis results of the defined samples (phase 1), the cleaned sediment extracts (phase 2), and the complete sediments (phase 3), all partici-

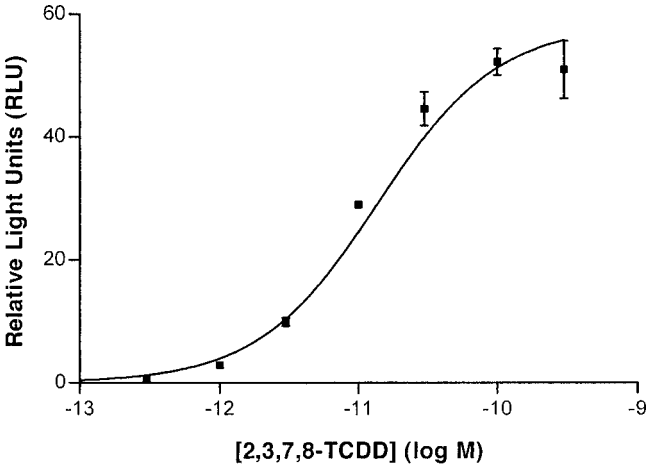

Fig. 1. Example of a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) standard calibration curve. The relative light units have been corrected for dimethyl sulfoxide blank. $r^2 = 0.993$.

pants also submitted the results of the complete 2,3,7,8-TCDD calibration curves for statistical evaluation.

Calibration curves were fitted, and EC50 values were derived using the nonlinear regression package pro Fit 5.5 (QuantumSoft, Zürich, Switzerland). The results of the calibration curve measurements were fitted to a sigmoidal dose–response function of the following form with a slope factor of 1:

$$R = R_0 + \frac{R_{max} - R_0}{1 + 10^{(EC50 - \mathrm{Log}C)}}$$

with C = concentration, R = response, R_0 = control response, R_{max} = maximum response, and EC50 = the concentration at which 50% response is observed. The R_0 was fixed in all fits to the response of the control sample at concentration 0; R_{max} and EC50 were fit by a nonlinear least-squares algorithm (the default Levenberg–Marquardt algorithm).

Analysis of variance (ANOVA) analyses were performed using the general statistical package StatView 5.01 (SAS Institute, Cary, NC, USA). The ANOVAs were calculated as repeated-measures ANOVAs with wells as within factor for phase 1 and with plates as within factor for subsequent phases. Specialized statistics, such as comparison of fits of different calibration curves, were calculated in MATLAB 5.1 (MathWorks, Natick, MA, USA) using custom routines.

RESULTS

Intralaboratory study

For the determination of the limit of detection (LOD) and LOQ, 10 standard 2,3,7,8-TCDD calibration series were analyzed in triplicate using the DR CALUX bioassay. In Figure 1, a typical example of a standard 2,3,7,8-TCDD calibration curve is given. For each individual calibration curve, the LOD was calculated as three times the standard deviation of the DMSO blank (0 pM 2,3,7,8-TCDD), whereas the LOQ was calculated as 10 times the standard deviation of the DMSO blank [26]. For the 10 standard 2,3,7,8-TCDD calibration curves, the LOD varied between 0.04 and 0.25 pM 2,3,7,8-TCDD per well. The LOQ varied between 0.12 and 0.88 pM 2,3,7,8-TCDD per well. Finally, an overall LOD and LOQ was calculated as the average of 10 observations plus three times the standard deviation (95% confidence) resulting in a LOD and LOQ of 0.3 and 1 pM 2,3,7,8-TCDD per well, respectively.

Table 1. Intralaboratory repeatability and reproducibility of the dioxin response–chemically activated lucerferase (DR CALUX®) bioassay for sediment extracts; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD); TEQ = toxic equivalent

	DR CALUX analysis result (pg 2,3,7,8-TCDD TEQ/g sediment)			
	Repeatability[ab]		Reproducibility[ab]	
	Sediment 1	Sediment 2	3 pM 2,3,7,8-TCDD	Sediment 4
Average	4.03	25.73	2.96	23.06
SD	0.97	2.54	0.41	4.58
%SD	24.1	9.9	13.8	19.9

[a] For the determination of the repeatability and reproducibility, each sample/extract analysis was analyzed 10 times in triplicate.
[b] The repeatability and reproducibility are calculated as percentage standard deviation (%SD).

For the determination of the repeatability, two sediments originating from coastal areas along the Dutch coastline were extracted and cleaned up. One of the sediments had a low 2,3,7,8-TCDD TEQ content, whereas the second sediment had a relatively high 2,3,7,8-TCDD TEQ content (4.8 and 26 pg 2,3,7,8-TCDD TEQ/g sediment, respectively). The 2,3,7,8-TCDD TEQ content in both extracts was determined by DR CALUX analysis 10 times on the same day. The reproducibility was determined by analyzing a 3-pM 2,3,7,8-TCDD standard and a cleaned sediment extract. Both samples were analyzed on 10 different days and by various persons. The results are summarized in Table 1.

The linearity of response of the bioassay depends on the linearity of the luminometer used. To determine the linearity of response, a concentration range of luciferase was prepared and the activity measured. A good linear correlation between the detected amount of light and the luciferase concentration was found ($r^2 = 0.9997$).

Interlaboratory study

Phase 1: 2,3,7,8-TCDD calibration curves. In phase 1 of the ring test, all six laboratories analyzed two calibration curves per microtiter plate, a BDS-supplied calibration curve, as well as a calibration curve prepared in house by the participants themselves. In Table 2, the EC50 values and the coefficients of determination for the curve fits for all participants are summarized. In addition, the 3-pM point of the 2,3,7,8-TCDD calibration curve is given. Differences in EC50 values reported by the participating laboratories are apparent. In particular, participant B reported relatively high EC50 values in both the calibration series provided by the coordinator and the calibration series prepared by participant B themselves.

Both the EC50 values and the 3-pM point of the 2,3,7,8-TCDD calibration curve serve as quality criteria. For each participant, the results for both data points from all 96-well plates analyzed during the presented study were collected and recorded in Shewhart control charts. The Shewhart control chart is used to identify variations on performance of the DR CALUX bioassay brought about by unexpected or unassigned causes. The Shewhart control chart shows the mean of the EC50 and 3-pM control point and the upper and lower control limits. In Figure 2, a typical Shewhart control chart is shown. Over the analysis period, none of the participants exceeded the action levels (AVG $\pm$ 3·S).

The results of the multiple analysis of the standard 2,3,7,8-

Table 2. Summary of dioxin responsive–chemically activated lucerferase (DR CALUX®) analysis result (pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [2,3,7,8-TCDD]/well) for the 2,3,7,8-TCDD calibration curves

	Calibration curve provided by coordinator		Calibration curve prepared by participant		
Participant	EC50[a] (pM TCDD/well)	r^2	EC50 (pM TCDD/well)	r^2	3 pM[b] (pM/well)
A	6.22	0.997	8.88	0.997	2.80
B	21.7	0.994	26.9	0.999	2.44
C	11.0	0.999	9.43	0.998	3.02
D	13.9	0.999	12.6	0.988	3.04
E	10.0	0.999	17.1	0.997	2.98
F	10.9	0.960	12.5	0.986	3.10

[a] Median effective concentration.
[b] Result of the 3 pM 2,3,7,8-TCDD calibration concentration prepared by the participants.

TCDD calibration curves are also be used to determine the per-participant LOD and LOQ taking into account interlaboratory variation (Table 3). This results show that on average, the participants of the calibration study meet the set LOD and LOQ derived from the intralaboratory study.

Phase 1: Defined standard solutions. Participants were asked to measure the response of the two standard samples in the DR CALUX bioassay three times in triplicate. The total DR CALUX 2,3,7,8-TCDD TEQ content of both the 2,3,7,8-TCDD sample as well as the mixed sample was calculated to be 7.5 nM TEQ. Since the DMSO content during exposure was 0.4% and the samples were diluted seven times by all participants, the expected DR CALUX TEQ content per well for both samples was 4.3 pM 2,3,7,8-TCDD TEQ. Overall, 27 individual measurements per sample and per participant were available for data analysis. Averaged results for the concentration of dioxin equivalents per participant and per sample are summarized in Table 4. The DR CALUX results for the dioxin sample are slightly higher than the actual 2,3,7,8-TCDD concentration in the sample. The results for the TCDD/PCB mixed sample are on average lower than the 2,3,7,8-TCDD TEQ content as calculated using CALUX REP values for the individual congeners.

Phase 2: Sediment extracts. In phase 2, participants were provided with three extracted sediment samples, all originating from the same batch. The participants were advised to dilute the supplied samples 10× and 30×. Since the three samples were analyzed on three separate plates, nine measurement values per extract dilution were available per participant. The ANOVA results for the sediment extract samples, when analyzed by individual participant, show that significant differences exist between the results obtained per laboratory ($p < 0.0001$) and also between the two dilutions employed ($p = 0.0007$). It was observed that DR CALUX analysis of the 30× diluted samples give higher results than the 10× diluted samples (data not shown). Averaged results for the concentration of 2,3,7,8-TCDD TEQs per participant and per sample (30× diluted) are given in Table 4. Quantitatively Bonferroni–Dunn multiple comparisons indicate that overall (taken over both dilutions), participant C is significantly different from the rest. Although not traceable anymore, and because the results obtained from the three control samples (DMSO blank, 3-pM 2,3,7,8-TCDD control, and internal reference sample) complied with the quality performance criteria for the DR CALUX bioassay, the indication is strong that a dilution error was made by participant C in the supplied sample.

In addition to the sediment extracts, all participants received a procedure blank. The procedure blank is analyzed to check for possible contamination from chemicals and materials used during extraction and/or cleanup. DR CALUX analysis results from this procedure blank for all participants were below the limit of quantitation (1 pM 2,3,7,8-TCDD TEQ/well) and therefore comply with the DR CALUX performance criteria (data not shown).

Phase 3: Sediment sample. In phase 3, participants were provided with a single contaminated sediment. The participants were asked to extract and clean up the sediment in three separate sessions using the Soxhlet extraction method. Following extraction and cleanup, the three sediment extracts were analyzed using the DR CALUX bioassay method. Participants were left free to choose their own dilutions. The different dilutions chosen made it impossible to perform variance analyses by participants × dilutions. Analysis of variance by par-

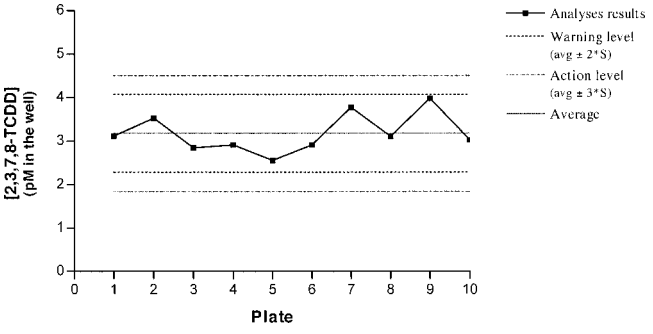

Fig. 2. Typical example of a Shewart control chart of the 3-pM point of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) calibration curve.

Table 3. Averaged limits of detection (LOD) and limits of quantitation (LOQ) (pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [2,3,7,8-TCDD]/well) over all experiments, by participant; SD = standard deviation; %SD = percentage standard deviation

	LOD		LOQ	
Participant	Average	SD	Average	SD
A	0.17	0.09	0.52	0.31
B	0.41	0.47	0.92	0.92
C	0.36	0.33	0.95	0.85
D	0.29	0.07	0.75	0.19
E	0.43	0.37	1.16	0.91
F	0.21	0.14	0.61	0.28
Average	0.31		0.82	
SD	0.11		0.24	
%SD	34.8		28.8	

Table 4. Dioxin responsive–chemically activated lucerferase (DR CALUX®) repeatability and reproducibility for the bioanalysis of the defined standard solutions and sediment samples of the various phases of the present interlaboratory validation study

Participant	Dioxin sample		Mixed sample		Sediment extract		Sediment	
	TCDD TEQ (pM)[a]	Repeatability (%SD)[b]	TCDD TEQ (pM)[a]	Repeatability (%SD)[b]	TCDD TEQ (pg/g)[a]	Repeatability (%SD)[b]	TCDD TEQ (pg/g)[a]	Repeatability (%SD)[b]
A	4.5	9.4	3.7	12.1	41.5	17.1	5.2	8.3
B	4.5	21.0	3.2	13.9	38.8	19.4	2.8	20.6
C	5.1	8.4	4.5	11.3	26.5	8.86	5.1	5.8
D	4.6	1.0	4.3	10.5	38.1	8.6	4.7	5.8
E1[c]	4.5	17.0	4.2	15.5	25.5	19.5	3.1	37.8
E2[c]	4.8	11.4	4.2	12.9	35.9	3.1	4.4	47.6
F	4.2	34.2	4.0	35.9	33.8	28.4	2.6	56.8
Average repeatability (%SD)		14.6		16.0		15.0		26.1
Average (pM)	4.6		4.0		34.3		4.0	
Reproducibility (%SD)	6.5		10.5		18.0		27.9	

[a] Data are expressed either as pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) toxic equivalents (TEQ)/well or as pg 2,3,7,8-TCDD TEQ/g extracted sediment (dry wt).
[b] The repeatability and reproducibility are calculated as percentage standard deviation (%SD).
[c] Participant E performed all DR CALUX analyses twice. Here, both reported results are taken into account.

Fig. 3. Averaged dioxin responsive–chemically activated lucerferase (DR CALUX®) results by participant for the sediment extracted by the participants (phase 3). Participants were free to choose their own dilution factor for analysis. Data presented are 0× to 200× diluted samples; 2,3,7,8-TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TEQ = toxic equivalents.

ticipants indicates that significant differences exist between laboratories (data not shown). Figure 3 shows graphical representations of the results by participants. The results are categorized as 0× to 200× dilutions. Since not all the participants submitted results in higher dilution ranges, these results were not evaluated. However, it should be noted that higher responses were observed by participants at higher dilutions as compared to the 0 to 200 category as presented here. In the lowest dilution category, significant differences ($p = 0.006$) exist between participants, mainly because of low analytical responses for participant F. Averaged results for the concentration of 2,3,7,8-TCDD TEQs per participant and per sediment are given in Table 4.

Repeatability and reproducibility. The repeatability of the DR CALUX bioassay was calculated for all samples (2,3,7,8-TCDD sample, mixed sample, sediment extract, and sediment sample) analyzed by the participants over the three phases of the interlaboratory validation study as the relative standard deviations of the obtained results (Table 4). The average repeatability for the participating laboratories ranged from 14.6% for the dioxin sample analysis to 26.1% for the sediment samples that had to be extracted by the participants themselves. It can be seen that the repeatability was lowest for phase 3, during which the participants were asked to extract, clean up, and perform a DR CALUX bioassay on a supplied sediment sample. In Table 4, the reproducibility for the various analyzed samples is also given. The percentage standard deviations over the DR CALUX bioanalysis results for the analyzed samples ranged from 6.5% for the dioxin sample to 27.9% for the supplied sediment sample. Again, the biggest differences in analysis results were observed in the sediment sample that had to be extracted and cleaned up by the participants themselves.

DISCUSSION

The aim of the present study was to identify the DR CALUX bioassay performance criteria for the analysis of PHAHs in sediment samples in order to implement the bioassay in the assessment of dredged materials for systematic monitoring in the coming years. Therefore, both an intra- and interlaboratory validation study was performed.

Standard 2,3,7,8-TCDD calibration curves

Calibration curves were made with a dioxin standard and were used to convert DR CALUX response levels to concentrations expressed as 2,3,7,8-TCDD TEQs. The section of the 2,3,7,8-TCDD calibration curve between the LOQ (1 pM) and the EC50 is used to quantify DR CALUX analysis results. This section is not linear (see Fig. 1). However, when the calibration curve is plotted on a linear–linear scale, the indicated region can be regarded as linear. In addition, the region between the LOQ and EC50 is chosen for quantification of analysis results since this region of the 2,3,7,8-TCDD calibration curve is least sensitive to variations in observed DR CALUX activity.

Because the 2,3,7,8-TCDD calibration curve is used for quantification of analysis results, the stability and quality of the calibration curves is important. Furthermore, the calibration curves themselves are used as a DR CALUX bioassay quality criterion. According to the performance criteria set for the DR CALUX bioassay, the fitted EC50 should be within the range of 6 to 18 pM 2,3,7,8-TCDD; otherwise, the results are rejected. In addition, the EC50 value of 2,3,7,8-TCDD should be constant over a longer time period. Finally, the coefficient of determination (r) should be more then 0.95 [27]. The numerical results of the fit are summarized in Table 2.

Differences in EC50 values between labs are apparent. Since actual fits are based on log (concentration) values and therefore yield log EC50 estimates, differences in these estimates will be exaggerated when transforming these values to EC50s. In addition, high EC50 values reported by participant B are correlated to low relative responses at the low end of the concentration range, up to and including 3.0 pM (individual curve fits not shown). Based on the results of the present study, EC50 values may range between 8.3 and 18.1 pM (based on a relative error of 15.4%). In a number of previous studies, EC50 values in the same range as suggested previously were found [20,21,28–30]. For the moment, the EC50 value of the 2,3,7,8-TCDD calibration curve is used as a quality control for the 2,3,7,8-TCDD calibration curve. It can be observed that EC50 values may differ between persons performing the DR CALUX bioassay but also between analyses performed by a single person. However, fluctuating EC50 values do not interfere with the final results of a DR CALUX analysis, especially with data quantified below the EC50 of the standard curve. Observed differences in the 2,3,7,8-TCDD calibration curves occur mainly at the high response end of the calibration curves above the EC50. Since the high end of the calibration curve is not used for data interpolation, differences do not significantly influence analysis results. Most probably, the EC50 value is an indication of the quality or condition of the cells rather than a performance criterion. Despite differences between individual calibration curves, the coefficient of determination for the individual analyzed 2,3,7,8-TCDD calibrations curves is high, and the 3-pM 2,3,7,8-TCDD concentration of the 2,3,7,8-TCDD calibration curves prepared by the participants themselves showed good comparability between the participating laboratories. The 3-pM 2,3,7,8-TCDD concentration is used as quality control and is registered on a Shewhart control chart. The calculated average value was 2.91 pM (standard deviation = 0.21). The percentage standard deviation was calculated to be 7.2%.

WHO-TEFs versus DR CALUX-REPs

The WHO-TEF values are internationally accepted toxic equivalent factors for dioxins, furans, and dioxin-like PCBs, as stated by the WHO and derived from both in vivo and in vitro studies. The relative toxic potency of dioxins, furans, and dioxin-like PCBs, relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), can also be derived from analyzing the response elicited by various congeners using the DR CALUX bioassay. The potencies found using this method are expressed as CALUX REPs (CALUX relative potencies). The CALUX REP values are actual TEF values for the congeners in the DR CALUX bioassay and represent the total toxic potency of all congeners present that show affinity toward the Ah receptor. A number of authors have compared WHO TEFS and DR CALUX REPs [25,28,29]. Differences between WHO TEFs and DR CALUX REPs are apparent. As a consequence, DR CALUX TEQs differ from HRGCMS-analyzed 2,3,7,8-TCDD TEQs in a given sample because of the difference between WHO TEFS and DR CALUX REPs [24,31]. This was demonstrated for the mixed sample from phase 1 analyzed by the participants. Furthermore, differences in DR CALUX–derived TEQs and HRGCMS-derived TEQs can be a result of the fact that by using HRGCMS, only specified dioxin and/or dioxin-like compounds are determined. In contrast, all compounds showing affinity toward the Ah receptor are detected by the DR CALUX bioassay.

LOD and LOQ

The LOD and the LOQ of the DR CALUX bioassay were determined by analyzing 10 standard 2,3,7,8-TCDD calibration series. From these analyses, it was concluded that taking into account 95% confidence, a LOD and LOQ of 0.3 and 1 pM 2,3,7,8-TCDD per well, respectively, should be applied. Hence, in case 10 g of sediment are processed and analyzed using 0.4% of DMSO per well, the LOD and LOQ can be calculated to be 0.04 and 0.16 pg 2,3,7,8-TCDD equivalents per gram of sediment. Similar LOD and LOQ were reported by a number of authors [17,30].

The participants of the interlaboratory validation study also analyzed multiple standard 2,3,7,8-TCDD calibration curves. From these data, a per-participant LOD and LOQ could be determined. On average, the participants of the calibration study met the set LOD and LOQ derived from the intralaboratory study. Furthermore, analysis of variance indicated that no significant differences in LOD between laboratories could be identified.

Effect of dilutions

The sediment extracts were analyzed at 10× and 30× dilutions. Whereas the 10× diluted samples showed an average DR CALUX TEQ content over all participants of 27.3 ± 4.0 pg 2,3,7,8-TCDD TEQ/g sediment, the 30× diluted extract gave a DR CALUX response of 34.9 ± 6.1 pg 2,3,7,8-TCDD TEQ/g sediment. In general, an effect of dilution on the total DR CALUX TEQ content in sediment samples is observed. Although the exact nature for this observation is not known, it is hypothesized that this is due to the presence of various compounds in sediment extracts showing variable affinity toward the Ah receptor. Dose–response curves in the DR CALUX bioassay of individual compounds have been studied and showed obvious differences [25] both in maximum response and slope of the curve fit.

Repeatability

For the determination of the intralaboratory repeatability of the DR CALUX bioassay for sediment samples, two sed-

iment extracts were analyzed 10 times. Each analysis was performed in triplicate. As a prerequisite for a correct triplicate analysis, the percentage standard deviation in the triplicate determination should be below 15%. This is in accordance with the harmonized quality criteria for cell-based bioassay analyses of PCDDs/PCDFs in feed and food as formulated by Behnisch et al. [27] and as detailed in European Union directive 2002/69/EC and directive 2002/70/EC. The repeatability for the low-2,3,7,8-TCDD-content sediment extract was found to be 24.1% whereas in the high-content-sediment extract, the repeatability was shown to be 9.9%.

For each participating laboratory, the repeatability of the DR CALUX bioassay was calculated for the four samples (2,3,7,8-TCDD sample, mixed sample, sediment extract, and sediment sample) analyzed by the participants over the three phases of the interlaboratory validation study. The average repeatability for the participating laboratories ranged from 14.6% for the dioxin sample analysis to 26.1% for the sediment samples that had to be extracted by the participants. In an interlaboratory comparison exercise for the analysis of PCDD/PCDFs in digested sewage sludge using HRGCMS, relative standard deviations for standard solutions varied between 15 and 41% [32]. Similar ranges of relative standard deviations were reported in two other round-robin studies for standard solutions: 18 to 61% and 8 to 43% [33,34]. This indicates that the determination of dioxin-like activity in sediment using the DR CALUX bioassay is at least as consistent as the established HRGCMS methods. In addition, the results show that the intra- and interlaboratory repeatability is comparable. From the data it can also been seen that the repeatability was lowest for phase 3, during which the participants were asked to extract, clean up, and perform a DR CALUX bioassay on a supplied sediment sample. Since in the third phase, extra steps to the total procedure are introduced (extraction and cleanup), it is very likely that these add to the variability of the total process. Furthermore, as none of the participants had prior experience using the supplied extraction procedure, it can be anticipated that with increasing experience using the supplied extraction protocol, the repeatability will also increase.

Reproducibility

As with the determination of the intralaboratory repeatability, the intralaboratory reproducibility was determined by analyzing a cleaned sediment extract and a 3-pM 2,3,7,8-TCDD standard on 10 separate days and by multiple persons. The reproducibility for the 3-pM 2,3,7,8-TCDD standard was found to be 13.8%, whereas the reproducibility for the cleaned sediment extract was shown to be 19.9%. Since the observed reproducibilities are in the range of relative standard deviations for two sediment extracts analyzed in 10-fold on the same day (intralaboratory repeatability), the DR CALUX bioassay can be evaluated as a stable and robust bioanalytical tool.

The interlaboratory results obtained from the analysis of defined standard solutions, but also from the analysis of sediment extracts prepared either by the coordinator of the study or by the participants themselves, also provide a measure of the variation between laboratories. The results show that the interlaboratory reproducibility ranges from 6.5% for the defined dioxin sample to 27.9% for the sediment sample extracted by the participants themselves. As was mentioned before, the reproducibility for this last sample is relatively high and most presumably due to the introduction of extra handlings (extraction and cleanup) to the total procedure. In addition, the fact that not all the participants had prior experience with the extraction protocol to be used could have added to the increase in variability of the process. Furthermore, the dilution factor was not dictated. This also introduces a certain degree of variation. For the reproducibility of the DR CALUX bioassay itself and not caused by differences in operating extraction conditions, the maximum variation between laboratories was observed to be 18.0%. The results for the sediment extract samples can also be used to estimate the method variability for extracts, that is, based on samples of unknown composition. Again, given the intra- as well as the interlaboratory variations observed in this study, it appears justified to conclude that the standard deviation of the means provides a reasonable estimate of the method variability, based on the overall average concentrations (in 2,3,7,8-TCDD TEQs) for a single sediment extract sample, determined at two different dilutions. The largest standard deviation of the means is therefore proposed as the method error for analyzed samples, being 18.0% for sediment extracts and 10.5% for analytical samples.

CONCLUSION

Several overall conclusions can be drawn based on the statistical evaluation of the data submitted by the participants of the DR CALUX intra- and interlaboratory validation study. First, differences in expertise between the laboratories are apparent based on the results for the calibration curves (both for the curves as provided by the coordinator and for the curves that were prepared by the participants) and on the differences in individual measurement variability. Second, the average results, over all participants, are very close to the ''true'' concentration, expressed in DR CALUX 2,3,7,8-TCDD TEQs for the analytical samples. Furthermore, the interlaboratory variation for the different sample types can be regarded as estimates for the method variability. The analytical method variability is estimated to be $\pm$10.5% for analytical samples and $\pm$22.0% for sediment extracts. Finally, responses appear dependent on the dilution of the final solution to be measured. This is hypothesized to be due to differences in dose–effect curves for different dioxin responsive element–active substances. For 2,3,7,8-TCDD, this effect is not observed. Overall, based on bioassay characteristics presented here and harmonized quality criteria published elsewhere [27], the DR CALUX bioassay is regarded as an accurate and reliable tool for intensive monitoring of coastal sediments.

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