Screening of Dioxin-Like Toxicity Equivalents for Various Matrices with Wildtype and Recombinant Rat Hepatoma H4IIE Cells

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Determination of dioxin-like compounds utilizing in vitro bioassays such as ethoxyresorufin-O-deethylase (EROD) or chemical-activated luciferase expression (DR-CALUX®) is an important tool to evaluate their Ah receptor-mediated toxic effects. The aim of this study is to describe advantages and limitations of these bioassays using rat hepatoma (H4IIE) wildtype and recombinant H4IIE cells in a 96 well microtiter plate format. We are using these bioassays for the evaluation of relative responses (REP) from several congeners of dioxins (e.g., 2,3,4,7,8-PCDF) or dioxin-like compounds (PCB-126, 2,3,4,7,8-PBDF) to 2,3,7,8-TCDD. In addition, total toxic equivalency factors (TEFs) of mixtures of these dioxin-like compounds from several kinds of matrices such as feed, sediment, or thermal waste residues are measured by both bioassays and additional chemical analysis. These samples were measured in a cross-validation study between two laboratories using the DR-CALUX technology in comparison to the H4IIE-EROD assay and chemical analysis. Improvement of the quality criteria of the newly developed DR-CALUX bioassays in comparison to the EROD bioassay was demonstrated (higher coefficient of determination \(r^2\); better repeatability of TCDD and samples), while induction factor, limit of detection, and limit of quantification have been similar. The tested samples showed positive responses in both bioassays using different kinetics (EROD: 72 h; DR-CALUX: 24 h). Ratio of measured toxicity equivalent (TEQ) values varied around mean values of 0.89 (comparing both DR-CALUX laboratories, ranging from 0.68–3.1), 2.0 (comparing EROD and DR-CALUX, ranging from 0.57–8.1), and 1.6–2.5 (comparing EROD-TEQ and I-TEQ, mean 1.6, ranging from 1.0–3.9; for DR-CALUX/I-TEQ, 2.5; 0.61–8.3), respectively. This demonstrates that these bioassays can be used as alternative screening technology for monitoring I-TEQ values in various standards and matrices.

Key Words: Ah receptor; biomarker; CALUX; dioxins; EROD; feed; fly ash; HAH; TCDD; sediments; toxic equivalency factors.

Numerous environmental chemicals possess dioxin-like properties. The mechanism of action has been extensively studied over the past decades. At elevated doses, natural and environmental Ah receptor (AhR) agonists (such as dioxin-like compounds) are known to produce adverse effects on humans and wildlife. Sources of potential exposure to dioxin-like compounds have to be identified for risk and hazard assessment. Dioxin-like compounds have been intentionally manufactured (PCBs), occurred as unwanted by-products in chemicals, or have been formed in industrial processes (PCDD/Fs in thermal processes). These persistent toxic substances are now found in all kinds of compartments of the biosphere (e.g., sediments/soils, feed/food) and tend to accumulate in biota.

Dioxin-like compounds show similar properties and display a wide variety of toxic effects in mammals, birds, and fishes (van den Berg et al., 1998, 2000). Binding to the AhR offers the biochemical mechanism (see for review Behnisch et al., 2001a,b; Brouwer et al., 1995; Hilscherova et al., 2000; Hoogenboom et al., 1999; Safe 1993, 1998a,b; Seidel et al., 2000; Ziccardi et al., 2000) to analyze the dioxin-like activity of these compounds quantitatively in a biological manner. Advantages are the extreme sensitivity, rapid, easy clean-up/work-up, small sample size, and reduced cost compared to instrumental methods. These bioassays are more biologically relevant than instrumental techniques. They have already been applied to a wide variety of matrices (food/feed, environmental samples, pure chemicals, waste recycling, and others) and have been recently reviewed by us (Behnisch et al., 2001a,b).

The approach in this article was to compare the accuracy and precision of two H4IIE cell bioassays, the first based on ethoxyresorufin-O-deethylase (EROD) activity and the second based on AhR-dependent luciferase reporter gene (DR-CALUX).

From both bioassays DR-CALUX technology is reported to be faster (in vitro luciferase: 4–48 h; EROD assay usually 24–72 h), less susceptible to interferences, with a better linear working range because of the instrument used (luminometer versus fluorometer). Therefore, recombinant cells exhibit greater sensitivity, dynamic range, and selectivity than wild-type cells (e.g., Garrison et al., 1996, Sanderson et al., 1996). The EROD bioassay using rat hepatoma H4IIE cell line is
already extensively used for measuring dioxin-like activity (e.g., Engwall and Hjelm, 2000; Gale et al., 2000; Hanberg et al., 1991; Li et al., 1999; Petrulis and Bunce, 1999; Safe, 1993; Sanderson et al., 1996; Schmitz et al., 1996; Schramm et al., 2001; Schrenk et al., 1991; Schweitzer et al., 1998; Till et al., 1997) and in an earlier study we validated this bioassay in comparison with the laboratory of GSF in Neuherberg, Germany (Behnisch et al., 2002). For the validation of the DR-CALUX technology we report here a cross-validation study with the laboratory of BioDetection-Systems, Amsterdam, The Netherlands, measuring several kinds of samples. The aim was to evaluate possible differences of several parameters such as coefficient of determination, limit of detection, and quantification, as well as the induction factor. These parameters will vary with the analytical method used (different luciferase kit and luminometer), the nature of the sample matrix (therefore we compared matrices as much as possible), and the specific congener/mixture measured. Recently, we started the discussion about quality criteria for measuring dioxin-like compounds (Behnisch et al., 2001c). We compare some of these quality criteria with the EROD-bioassay using H4IIE-wildtype cells. Finally, this study compares toxicity equivalent (TEQ) levels measured by EROD bioassay and chemical analysis as well as by DR-CALUX bioassay in two laboratories in Japan and the Netherlands by the same operator.

MATERIALS AND METHODS

Chemicals and samples. This study was started in June 2000 analyzing three standards (PCB-126, 2,3,4,7,8-PCFD, 2,3,4,7,8-PCDF), 4 mixtures of PCBs/PCDD/Fs, and 13 sample extracts of fly ashes (n = 3), emission gas (n = 1), PCB capacitor oils (n = 2), mineral oil (n = 1), sediments (n = 3), and feed stuffs (n = 3) at BioDetection Systems (BDS, Amsterdam) and was repeated later at Kaneka Corporation (KC; Takasago, Japan).

Clean-up of sample extracts. Fly ash samples 1 and 2 were cleaned up by a sandwich column (with sulfuric acid) and an alumina column, while the combustion sample was only passed through a simple silica gel column as reported earlier (Behnisch et al., 2002).

In the case of PCB capacitor and mineral oils 0.1 g of sample was exchanged at first by liquid/liquid from paraffin oil to DMSO and then additionally cleaned up by a silicagel (1 g/silicagel-H2SO4, 22%) column (4 g) and 50 ml n-hexane elution as described earlier (Sakai et al., 2001).

Fly ash, feed, and sediments were cleaned up by a mixed 20% and 30% sulfuric acid/silicagel (each 5 g) column and eluted with 40 ml hexane/diphenyl ether (97:3) according to BDS guidelines.

Bioassay Procedure

H4IIE-EROD assay. The Micro-EROD bioassay with rat hepatoma H4IIEC3/T cells were performed as described earlier (Behnisch et al., 2001c). Briefly, cells were cultured in α-MEM medium (GibcoBRL 14501-029) supplemented with 10% v/v FCS (JRHI12013-7SP) under standard conditions (37°C, 5% CO2). Cells were seeded into 96 well cell culture plates (Iwaki, Japan) at a density of 0.4–1 × 104 cells/well. After three days of growth (density of about 70–90% confluency), TCDD (0.3–300 pM, Cambridge Isotope Laboratories) or test-material was added in 200 µl FCS containing medium. All TCDD standards or samples were dissolved in DMSO (Djojin, Wako, Japan) and were added to the cell cultures in triplicate. The final concentration of solvent in the medium was 0.4%. The cells were exposed for another 72 h. 2,3,7,8-TCDD and the sample were simultaneously analyzed in a minimum of 5 concentrations in comparison to a blank sample on each 96 well plate. Then, the medium was removed and 100 µl fresh medium containing 16 µM 7-ethoxyresorufin (Sigma E3763), and 10 µM dicumarol (Sigma M1390) were added. After incubation at 37°C for 60 min, 90 µl of the reaction mixture was transferred to another 96 well plate containing 130 µl methanol. Resorufin-associated fluorescence was measured at 550 nm excitation and 585 nm emission using a multiwell fluorescence reader (Corona MTP-32 and MTP-F2). Measured TCDD-EQ values were unaffected by the protein content per well, which therefore was not included in the calculation.

DR-CALUX assay (BDS, Amsterdam, The Netherlands). In the case of the DR-CALUX bioassay performed at BDS, the validation samples were analyzed according to their guidelines (www.biotedetectionsystems.com) and recently published studies (Hamers et al., 2000; Koppen et al., 2001; Pauwels et al., 2000). At the BDS laboratory the glow mix was prepared by using Luciferin from Duchefa, and the induction was measured by luminometer from Anthos, Lucy 1 (software: version 1.5 and microwin version 3.0).

RESULTS AND DISCUSSION

Studies in other laboratories have already compared the CALUX and EROD bioassay using the rat hepatoma H4IIE cell line (Merk et al., 1996; Sanderson et al., 1996). However, the purpose of our study was to evaluate the reliability and ease of handling of this method for the establishment of a possible high-throughput screening technology in the future. For assay optimization several fetal calf serum, kinetics, 96 well plates, TCDD standards, incubation times for LucLite, and calculation methods were tested in our laboratory during the establishment period of the DR-CALUX bioassay.
Quality Criteria

In order to evaluate the performance of the DR-CALUX bioassay in the laboratory of KC, quality criteria were compared to our study earlier at the laboratory of BDS. These results in Table 1 and Figure 1 show that similar quality criteria have been obtained between both laboratories performing the DR-CALUX bioassay.

Compared to the EROD bioassay we obtained a comparable sensitivity (limit of detection around 0.30 pM; limit of quantification around 1 pM) and linear working range (0.3–30 pM).

The primary improvement of the CALUX assay compared to the EROD assay, however, is that the CALUX assay is more reliable as indicated by the repeatability of TCDD (DR-CALUX, 14–18%; EROD bioassay, 31%), repeatability of the tested samples (DR-CALUX, 18–20%; EROD bioassay, 26%), and the quality of the dose-response curve expressed by the coefficient of determination $r^2$ (DR-CALUX, 0.98–0.99; EROD bioassay, 0.96).

Cross-Validation Study for the DR-CALUX Bioassay

At first, the DR-CALUX technology was validated with 2,3,7,8-TCDD in a cross-validation study with BDS. The EC$_{50}$ value (in pM and the coefficient of variation [CV]), correlation coefficient ($r^2$) value of the EC$_{50}$ curve, minimal quantification limit (MQL), and minimal detection limit (MDL) of 2,3,7,8-TCDD analyzed by the DR-CALUX bioassay in the cross-validation study with BDS were comparable, e.g. (EC$_{50}$: 10 pM/ MQL: 1.0 pM/ MDL: 0.29 pM [n = 18]); reported by Bovee et al., 1998 (EC$_{50}$: 7 pM, limit of detection: 0.27 pM); Murk et al., 1996 (EC$_{50}$: 10 pM) and in the present study analyzed at KC (EC$_{50}$: 14 pM; CV:14%; MQL: 1.1 pM; MDL: 0.30 pM; [n = 18, independent tests]).

Several PCB/PCDD/F congeners showed similar REP values analyzed in the two laboratories by DR-CALUX bioassay and in addition by the Micro-EROD bioassay (see Table 2 and Fig. 2). REP values for 2,3,4,7,8-PCDF and PCB-126 measured by DR-CALUX have been similar to earlier publications (Bovee et al., 1998; Laier et al., 2001; Sanderson et al., 1996: 2,3,4,7,8-PCDF, 0.34, 0.69, and 0.51, respectively, versus our study, 0.75; and PCB-126, 0.065, 0.017, and 0.28, respectively, versus our study, 0.072).

TEQ values of several mixtures of PCBs and PCDD/Fs measured by the DR CALUX-bioassay in these two laboratories, were in the expected range. Additionally, the values analyzed by Micro-EROD and calculated I-TEQ values (based on the WHO-TEFs, 1997) were comparable.

Bio- and I-TEQ Values: CALUX and EROD Bioassays versus I-TEQ Values Measured by Chemical Analysis

In the second step, different matrices such as PCB oils, mineral oils, combustion gas, fly ashes, foodstuffs, and environmental (sediments) samples were analyzed by the DR-CALUX-technology in both laboratories. Several samples were additionally measured by the Micro-EROD bioassay and/or chemical analysis (Table 2, Fig. 3). The ratio of the measured TEQ values varied around a mean value of 0.89 (comparing both DR-CALUX laboratories, ranging from 0.68–3.1), 2.0 (comparing EROD and DR-CALUX, ranging from 0.57–8.1) and 1.6–2.5 (comparing EROD-TEQ and I-TEQ).

### TABLE 1

<table>
<thead>
<tr>
<th>Quality criteria</th>
<th>H4IIE-luc (KC)</th>
<th>H4IIE-luc (BDS)</th>
<th>H4IIE-EROD (KC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of tests</td>
<td>18</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Sum of measurements</td>
<td>320</td>
<td>18</td>
<td>450</td>
</tr>
<tr>
<td>EC$_{50}$ (pM)</td>
<td>14</td>
<td>10</td>
<td>5.0</td>
</tr>
<tr>
<td>Repeatability of TCDD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% SD of EC$_{50}$</td>
<td>14</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td>Coefficient of determination $r^2$</td>
<td>0.984</td>
<td>0.99</td>
<td>0.955</td>
</tr>
<tr>
<td>Limit of detection (pM)</td>
<td>0.30</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>Limit of quantification (pM)</td>
<td>1.1</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Repeatability of samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% SD of TEQ</td>
<td>20</td>
<td>18</td>
<td>26</td>
</tr>
<tr>
<td>Induction factor</td>
<td>8.8</td>
<td>6.9</td>
<td>9.7</td>
</tr>
</tbody>
</table>

Note. SD, standard deviation; KC, Kaneka Corporation; BDS, BioDetectionSystems.
TABLE 2
Cross-Validation Study between Two Laboratories Using the DR-CALUX Bioassay in Comparison to H4IIE-EROD and Chemical Analysis

<table>
<thead>
<tr>
<th>Samples</th>
<th>KC (A) DR-CALUX [n; CV]</th>
<th>BDS (B) DR-CALUX [n; CV]</th>
<th>Ratio A/B</th>
<th>KC (C) Micro-EROD [n; CV]</th>
<th>WHO-TEF or TEQ</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,7,8-PCDF</td>
<td>0.75 ± 0.06 [4; 8]</td>
<td>0.84 ± 0.16 [3; 23]</td>
<td>0.89</td>
<td>0.41 ± 0.17 [5; 42]</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>2,3,4,7,8-PBDF</td>
<td>0.069 ± 0.022 [5; 32]</td>
<td>0.99 ± 0.017 [2; —]</td>
<td>0.70</td>
<td>0.055 ± 0.021 [5; 38]</td>
<td>—</td>
<td>1.3</td>
</tr>
<tr>
<td>PCB-126</td>
<td>0.072 ± 0.014 [6; 19]</td>
<td>0.073 ± 0.016 [3; 22]</td>
<td>0.99</td>
<td>0.049 ± 0.018 [6; 36]</td>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Dioxin mixture (EPA-1613)</td>
<td>260 ± 39 [16; 15]</td>
<td>2900 [2; —]</td>
<td>0.90</td>
<td>310 ± 94 [9; 30]</td>
<td>240</td>
<td>0.84</td>
</tr>
<tr>
<td>PCB mixture</td>
<td>98 ± 21 [3; 21]</td>
<td>140 [2; —]</td>
<td>0.70</td>
<td>N/A</td>
<td>160</td>
<td>—</td>
</tr>
<tr>
<td>Co-PCB mixture</td>
<td>270 ± 59 [11; 22]</td>
<td>330 [2; —]</td>
<td>0.82</td>
<td>260 ± 75 [13; 29]</td>
<td>240</td>
<td>1.0</td>
</tr>
<tr>
<td>PCBs/PCDD/Fs</td>
<td>160 ± 32 [7; 20]</td>
<td>150 [1; —]</td>
<td>1.10</td>
<td>210 ± 51 [8; 24]</td>
<td>150</td>
<td>0.76</td>
</tr>
<tr>
<td>2,2,4,4’,5’-PBDE</td>
<td>&lt; 4.2 × 10⁻⁴</td>
<td>&lt; 4.2 × 10⁻⁴</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Combustion gas</td>
<td>72 ± 11 [6; 15]</td>
<td>97 ± 14 [3; 14]</td>
<td>0.74</td>
<td>8.9 ± 2.6 [5; 29]</td>
<td>8.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Fly ash 1</td>
<td>130 ± 13 [5; 10]</td>
<td>130 ± 10 [3; 8]</td>
<td>1.0</td>
<td>51 ± 6.7 [7; 14]</td>
<td>44</td>
<td>4.6</td>
</tr>
<tr>
<td>Fly ash 2</td>
<td>17 ± 3.0 [6; 18]</td>
<td>14 ± 1.4 [3; 10]</td>
<td>1.2</td>
<td>7.4 ± 1.4 [7; 14]</td>
<td>5.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Fly ash 3</td>
<td>210 ± 36 [5; 17]</td>
<td>240 ± 26 [3; 11]</td>
<td>0.88</td>
<td>370 ± 150 [3; 42]</td>
<td>95</td>
<td>0.57</td>
</tr>
<tr>
<td>PCB oil 1</td>
<td>2300 ± 370 [10; 16]</td>
<td>2100 [2; —]</td>
<td>1.1</td>
<td>1500 ± 180 [3; 12]</td>
<td>1150</td>
<td>1.5</td>
</tr>
<tr>
<td>PCB oil 2</td>
<td>0.36 ± 0.05 [6; 15]</td>
<td>0.36 [2; —]</td>
<td>1.2</td>
<td>0.059 [4; 33]</td>
<td>61</td>
<td>3.6</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>2240 ± 580 [6; 26]</td>
<td>2750 [2; —]</td>
<td>0.81</td>
<td>710 ± 310 [3; 44]</td>
<td>4.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Feed sample 1</td>
<td>2.9 ± 0.84 [9; 29]</td>
<td>3.5 ± 0.84 [6; 24]</td>
<td>0.83</td>
<td>2.7 ± 0.12 [3; 4]</td>
<td>4.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Feed sample 2</td>
<td>220 ± 49 [7; 22]</td>
<td>230 ± 55 [3; 24]</td>
<td>0.96</td>
<td>220 ± 55 [3; 25]</td>
<td>4.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Sediment 1</td>
<td>0.055 ± 0.02 [8; 37]</td>
<td>0.063 ± 0.011 [3; 17]</td>
<td>0.87</td>
<td>0.027 ± 5.5 [3; 20]</td>
<td>2.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Sediment 2</td>
<td>0.080 ± 0.021 [8; 26]</td>
<td>0.26 ± 0.096 [3; 37]</td>
<td>3.1</td>
<td>0.13 ± 0.015 [3; 11]</td>
<td>0.62</td>
<td>0.3</td>
</tr>
<tr>
<td>Sediment 3</td>
<td>0.13 ± 0.02 [6; 17]</td>
<td>0.19 [2; —]</td>
<td>0.94</td>
<td>0.086 ± 0.022 [3; 26]</td>
<td>4.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Mean value</td>
<td></td>
<td></td>
<td>0.89</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. KC, Kaneka Corporation. CV, Coefficient of variation (%).

* N/A, not available.

a TEF.

b TCDD-EQ.
c TEQ-calculated (pg TEQ/ml).
d TEQ-measured (PCBs + PCDD/Fs; ng-TEQ/ml, g or m³).

I-TEQ, mean 1.6, ranging from 1.0–3.9; for DR-CALUX/I-TEQ, 2.5; 0.61–8.3), respectively. It was concluded on the basis of these observations that the TEQ values obtained by both bioassays by complex mixtures of polyhalogenated aromatic hydrocarbons (PAHs) are well predicted by the TEF concept.

A remaining question is if, on the basis of these results, it can be concluded whether the current congener specific approach for evaluation the TEQ values and the risk estimation of complex mixtures of PCDDs, PCDFs, and PCBs is appropriate or not. On the basis of the dose-response curves it can be assumed that in the case of the here-tested PCB-126, 2,3,4,7,8-PCDF, and 2,3,4,7,8-PBDF, similar shapes have been obtained (% TCDD of PCB-126, around 100%; 2,3,4,7,8-PCDF, around 110%; and 2,3,4,7,8-PBDF, around 100%) making the WHO proposed TEF values reasonable (van den Berg et al., 1998).

In the case of the mixtures of PCBs and PCDD/Fs using the linear part of the dose-response curve for the calculating of TEQ values compared to concentration based I-TEQ values weak synergistic effects were obtained, confirming earlier results (Schrenk et al., 1991).

For complex industrial samples primarily polluted by PCDD/Fs (such as fly ash and emission gas), the TCDD-EQ are well correlated with the I-TEQ, due to a similar shape of the dose-response curve of TCDD (Fig. 3).

The tested feed samples showed similar TEQ values in both bioassays. In the case of the sediment samples the comparison of CALUX-TEQ and EROD-TEQ ranged between 0.6–2.0.

In the case of PCB-oils (as weak agonists), it remains an open question if it will be possible to analyze such complex mixture (with antagonistic and agonistic effects; see Murk et al., 1996; Petruulis and Bunce, 1999; Schmitz et al., 1996; Tysklind et al., 1995) with Ah receptor based cell bioassays.
Certainly, the separation of di-ortho-PCBs from the dioxin-like PCB fraction is preferred and will be tested further in the future (van der Plas et al., 2000).

The use of the DR-CALUX system here described for the detection of Ah receptor agonists has many advantages over the EROD methods previously used. It is easier, more reliable, less time consuming, and fewer inhibition effects have been reported. Therefore, this bioassay could be a valuable new tool for rapid assessment of all kind of chemical standards or complex environmental pollutants. The wide possibility of application makes this tool very useful for biomonitoring studies as well as screening of industrial chemicals or processes. But in the future this system will also have to fulfil several quality criteria like the chemical analysis, which still has to be evaluated and applied on round robin studies. There is an urgent need to evaluate which clean-up should be used (with or without nondioxin-like compounds such as PAHs) and which compounds should be tested for their dioxin-like potency. This combination of fractionation and toxicological risk assessment will provide further information on which matrices need further consideration and which kind of potential exposure to any kind of AhR agonists we can expect.

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FIG. 2. Different shapes of dose-response curves of luciferase activity of several Ah receptor agonists measured by DR-CALUX (24 h kinetic) and EROD (72 h kinetic) bioassay: 2,3,7,8-TCDD (classic), 2,3,4,7,8-PBDF (classic), PCB-126 (steep), Benzo[al]pyrene (superinduction), and PBDE-126 (weak induction). Bioassays were performed as described in Materials and Methods. Values represent the means of at least a triplicate determination.

FIG. 3. Typical shapes of dose-response curves of luciferase activity of several tested samples analyzed by DR-CALUX bioassay (24 h kinetic): 2,3,7,8-TCDD, combustion gas, fly ash, transformer PCB-oil, feed, sediment. Bioassays were performed as described in Materials and Methods. Values represent the means of at least a triplicate determination.


