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Chemical-Activated Luciferase Gene Expression (CALUX): A Novel *in Vitro* Bioassay for Ah Receptor Active Compounds in Sediments and Pore Water

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This study demonstrates that the novel *in vitro* CALUX (chemical-activated luciferase expression) assay is a rapid, sensitive assay for assessing the toxic potency of (mixtures of) aryl hydrocarbon receptor (AhR)-active compounds in sediments and pore waters. A rat hepatoma (H4IIE) cell line, stably transfected with a construct containing the dioxin-responsive element (DRE) sequence and the luciferase reporter gene, was used to determine the relative potency or the total activities of AhR-active compounds in sediment and pore water extracts. This novel CALUX assay had a detection limit of 0.5 fmol of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The sensitivity and linear working range was slightly better than for the ethoxyresorufin *O*-deethylase (EROD) assay in H4IIE wild type cells. The primary improvement of the CALUX assay compared to the EROD assay, however, is that the CALUX assay is insensitive to substrate inhibition. The CALUX activity induced by organic extracts from 450-mg aliquots of sediment or 250-μl aliquots of pore water corresponded with the instrumentally analyzed degree of pollution of the sediment. Using pore water, only a simple and rapid extraction procedure was needed, without additional clean-up to prevent cell death. The response from pore water samples in an 8-day early life stage test with zebra fish (*Branchydanio rerio*) corresponded with the CALUX induction, although the correlation was sometimes disturbed by heavy metals. Two polychlorinated terphenyl mixtures, the PCB-substitute Ugilec 141, polybrominated diphenylethers, and the PCB-mixture Clophen A50 were tested in the CALUX assay and had induction potencies that were 10^{-4}–10^{-7} compared to TCDD. © 1996 Society of Toxicology

Sediments at many locations in Dutch waters are heavily polluted with polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorodibenzo-*p*-dioxins (PCDDs), -dibenzofurans (PCDFs), and -biphenyls (PCBs). Of the large group of PHAHs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical and most potent member. PHAHs are persistent and lipophilic and elicit a number of species-specific, toxic responses in vertebrates, including hepatoxicity, body weight loss, thymic atrophy, impairment of immune responses, dermal lesions, reproductive toxicity, alterations in vitamin A and thyroid hormone metabolism, teratogenicity, and carcinogenesis (Poland and Knutson, 1982; Goldstein and Safe, 1989; Brouwer, 1991; Giesy *et al.*, 1994; Safe, 1994). Studies with aquatic organisms have concentrated mainly on fish. They have been found to be very sensitive to PHAH-induced toxicity, especially when exposed during early life stages, i.e., egg to larval stadium (Kleeman *et al.*, 1986). Typical toxic responses are malformations, hemorrhages, and pericardial edema (Walker *et al.*, 1991, 1992). PHAH-contaminated sediments thus may pose a serious threat to aquatic species and their predators, including fish-eating birds, mammals, and humans. Therefore a rapid, inexpensive assay is needed for monitoring the toxic potency of a great number of samples. We feel that our novel *in vitro* bioassay, with chemical-activated luciferase expression (CALUX) based on the mechanism of action of PHAHs, will facilitate rapid assessment of the toxic potency of mixtures of PHAHs in environmental matrices.

BIOMARKERS FOR PHAHs

Of the hundreds of existing PHAHs, those with one or no *ortho*-substituted halogen can assume a planar configuration, making them approximate isostereomeres TCDD. The mechanism of action for these relatively toxic, planar PHAHs has been partially elucidated. After binding of the ligand to the cytosolar aryl hydrocarbon receptor (AhR), the ligand-recep-

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tor complex is activated and translocated to the nucleus, wherein it binds to a dioxin responsive element (DRE) and stimulate transcription of an adjacent gene (Denison and Whitlock, 1995). Examples of induced genes are the phase I and II enzymes cytochrome P450 1A and UDP-glucuronyltransferase (DeVito and Birnbaum, 1994). Although it is not known how cytochrome P450 1A activity contributes to specific PHAH toxicity, its associated increase in ethoxyresorufin *O*-deethylase (EROD) activity is often studied *in vivo* and *in vitro* because it is altered in tandem with that of other enzymes and receptor proteins (Brouwer, 1991; Tillitt *et al.*, 1991; Jones *et al.*, 1993; Eggens *et al.*, 1995). The EROD bioassay, however, poses some disadvantages, such as *in vivo* season-dependent fluctuations in inducibility, low enzyme stability after death of an animal, and *in vitro* inhibited activity at greater ligand concentrations (Kennedy *et al.*, 1993; Sawyer *et al.*, 1984; Hahn *et al.*, 1993).

In our laboratories, an additional *in vitro* bioassay for PHAHs has been developed, based on AhR-mediated firefly (*Photinus pyralis*) luciferase gene expression (Aarts *et al.*, 1995; Garrison *et al.*, 1996). A vector containing the luciferase gene under transcriptional control of DREs isolated from the 5-flanking region of the mouse P450 1A1 gene, was stably transfected into a number of hepatoma cell lines, including mouse (Hepa1c1c7) and rat (H4IIE) cell lines. The induction of luciferase activity in the transfected cells, upon exposure to TCDD, is dose-dependent and comparable to that of the natural cytochrome P450 1A activity (Aarts *et al.*, 1995; Garrison *et al.*, 1996; Sanderson *et al.*, 1996).

For hazard and risk assessment purposes of mixtures of PHAHs, the relative toxic potencies of individual PHAH congeners compared to TCDD have been transformed into toxic equivalency factors (TEFs). The concentrations of the individual congeners measured, multiplied by their respective TEFs, are added up to give the total TCDD toxic equivalency (TEQ) of the mixture (Safe, 1990; Ahlborg *et al.*, 1992). For the PCDD-, PCDF-, and PCB-congeners tested so far, the potency to induce CALUX activity relative to TCDD (CALUX-TEF) has been in accordance with reported TEF values (Denison *et al.*, 1993; Aarts *et al.*, 1995; Garrison *et al.*, 1996; Sanderson *et al.*, 1996).

THIS STUDY

In this paper we describe the use of the CALUX assay for monitoring AhR-active compounds associated with sediments. In addition to PCBs, PCDDs and PCDFs, other PHAHs, such as polychlorinated terphenyls (PCTs) (Watanabe *et al.*, 1987; De Boer, 1995), tetraclorobenzyltoluenes (the PCB-substitute Ugilec 141) (De Boer, 1995), and polybrominated diphenylethers (PBDEs, widely used as fire retardants) (Watanabe *et al.*, 1987; Andersson and Wartanian, 1992; IPCS, 1994; Pijnenburg *et al.*, 1995), will end up in the organic extracts. The relative potency of these PHAHs in the CALUX assay is compared to that seen with TCDD. Sediment samples from a number of locations in the Netherlands were collected, and extracts of both whole sediment and pore water were tested in the CALUX assay. The CALUX response of pore water extracts is compared to the biological response in an early life stage (ELS) test with the zebra fish (*Branchydanio rerio*).

MATERIALS AND METHODS

Chemicals

All chemicals used were of pesticide analysis (pa) or HPLC grade. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was purchased from Schmidt B.V (Amsterdam, The Netherlands). The technical brominated diphenyl ether mixture Bromkal 70-5-DE, and three pure congeners 2,2′,4,4′,5,5′-hexabromodiphenylether (HBDE), 2,2′,4,4′,5-pentabromodiphenylether (PBDE), and 2,2′,4,4′-tetrabromodiphelylether (TBDE) were provided by Dr. Åke Bergman (Environmental Chemistry, Stockholm University, Sweden). The technical PCB mixture Clophen A50, the PCT mixtures Aroclor 5442 and 5460, and the tetrachlorobenzyltoluene mixture Ugilec 141 were provided by Dr. De Boer (Netherlands Institute for Fisheries Research, RIVO, IJmuiden) GC-MS analysis to assure the purity and concentration of the TCDD stock solutions was performed by the Dutch State Institute for Quality Control of Agricultural Products (RIKILT-DLO). For exposure of cells, all chemicals were dissolved in ultraclean dimethylsulfoxide (DMSO; Janssen, Belgium).

CALUX Assay

For experiments with individual congeners, rat (H4IIE) or mouse (Hepa1c1c7) hepatoma cell lines, stably transfected with the luciferase (Luc) reporter gene plasmid pGudLuc1.1 were used (further called H4IIE.Luc and Hepa.Luc cells, respectively). These cell lines were prepared as previously described in Aarts *et al.* (1995) and Garrison *et al.* (1996) (also called H4L1.1c4 and H1L1 1c7). The cells were grown in 24-well cell culture plates (Costar) in 0 5 ml minimal essential medium (α-MEM, Gibco) with 10% heat-inactivated (h.i.) fetal calf serum (FCS, Gibco) or in 6-well cell culture plates (Costar) in 3 ml α-MEM with 10% h.i. FCS. The cell layer reached 90–100% confluence 24 hr after seeding, and growth medium was replaced with fresh culture medium containing test compounds or extracts in a maximum of 0.5% DMSO. Exposure was in triplicate, and for each assay a TCDD standard series from 1 to 1000 pM was included After 20–24 hr of exposure the medium was removed and cells washed twice with phosphate-buffered saline (PBS), and the cells were harvested in 250 μl (6-well plates) or 75 μl (24-well plates) cell lysis reagent (Promega), centrifuged for 3 min (6-well plates) or 90 sec (24-well plates) at 13,000*g* and the supernatant frozen at −80°C. For measurement of luciferase activity the samples were thawed on ice, 20 μl supernatant was pipetted into a 96-well microtiter plate, and 100 μl luciferin assay mix (Promega) at room temperature was added and the plate was mixed for approximately 90 sec on a plate mixer. The light production was measured in an Amerlite Luminometer (Amersham). For each sample the protein content was measured in a microtiter plate at 595 nm, according to Bradford (1976) using protein assay dye reagent (BioRad) and bovine serum albumin (BSA) as protein standard.

EROD Assay

The EROD activity was measured using 96-well microtiter plates, mainly based on the method described by Sanderson *et al.* (1996) Briefly, H4IIE or Hepa1c1c7 wild type cells were seeded in 96-well plates in 100 μl α-

MEM. After 24 hr incubation at 37°C the cell layer was 80–90% confluent, and 100 μl of fresh α-MEM containing the test compound in maximal 0.5% DMSO was added to each well. Samples were tested in four- or sixfold replicates. After an additional 48-hr incubation, the medium was removed and the wells were rinsed twice with 50% diluted PBS. To each well 20 μl of nanopure water was added to swell the cells and after 15 min incubation at room temperature, the plates were placed at −80°C to lyse the cells. To measure resorufin production, 50 μl of Tris–sucrose buffer (pH 8) with 40 μM dicumerol, followed by 25 μl 10 μM 7-ethoxyresorufin (ER) were added to each well and the plates were preincubated for 20 min at 37°C. To start the reaction, 25 μl 1 mM NADPH solution was added per well and the plates were again incubated, 1 hr at 37°C. The resorufin production was measured in a fluorometer (Cytofluor) with an excitation filter at 530 nm and an emission filter at 590 nm. Protein measurement was carried out in the same plates, using the Pierce method (Smith *et al.*, 1985), for which, unlike the Bradford method described above, dilution of samples was not necessary. Samples were left overnight at 37°C to dry until slightly wet. After addition of bicinchoninic acid (Pierce) followed by 30 min incubation at 37°C, the absorbance at 562 nm was measured in a microtiter plate spectrophotometer (Thermomax, Molecular Devices). BSA was used as protein standard.

Substrate Inhibition Test

For the CALUX inhibition assay, H4IIE.Luc or Hepa.Luc cells were exposed to 50 pM TCDD in 24-well plates for 24 hr. Upon harvesting and addition of lysis mix, the plates were frozen at −80°C and later thawed on ice prior to luciferase activity measurement. Final concentrations of Clophen A50 ranging from 0.1 to 500 μM and 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) ranging from 0.01 to 50 μM were reached by adding appropriate concentrations of stock solutions in 4 μl DMSO to the 20-μl cell lysate in each well. The plates were allowed to stand 5 min at room temperature and then thoroughly mixed for 5 min at 37°C on a plate mixer. Samples of 20 μl were measured for light output as described above. For the EROD assay, H4IIE or Hepa wild type cells were exposed to 50 pM TCDD in 96-well plates for 48 hr. Upon harvesting and addition of nanopure water, plates were frozen at −80°C and later thawed on ice for EROD measurement. Final concentrations of Clophen A50 ranging from 0.1 to 500 μM and PCB 77 ranging from 0.01 to 50 μM were achieved by adding stock solutions in 4 μl DMSO per well shortly after cell lysis at −80°C. The plates were then thoroughly mixed on a plate mixer for 20 min at 37°C, 50 μl of Tris–sucrose buffer (pH 8) with 40 μM dicumerol, followed by 25 μl 10 μM ER were added to each well, and the plates were preincubated for 20 min at 37°C To start the reaction, 25 μl 1 mM NADPH solution was added per well and resorufin production was measured as described above

CALUX Measurement of Sediment and Pore Water Extracts

Sediment samples. Sediment samples collected throughout the Netherlands were obtained from the National Institute for Inland Water Management (RIZA) from the following locations: Ketelmeer (KM1, KM3, and KM13), Oostvaardersplassen (OVP), North Sea Canal (NSC), Drontermeer (DM), Markermeer (MM), Brabantse Biesbosch (BB), Dommel (DOM), Delfland (DL), and Spijkerboor (SB). Sediments were sampled with an Eckman grab and stored in large plastic vials at 5°C. Of the sediment samples used, KM and OVP are routinely assayed in RIZA biomonitoring programs. KM, BB, and SB are sedimentation areas of the rivers Rhine and Meuse and known to be polluted with a wide range of chemicals, including PCBs and dioxins. The OVP is not in direct contact with one of the main rivers and is therefore considered to be a reference site (Maas *et al.*, 1993). Also MM, DM, and DL have low contamination levels in the sediment. NSC and DOM have regional inputs of organic micropollutants and heavy metals, respectively

Extraction and cleanup of sediment. Sediment samples were homogenated, extraneous overlying water was decanted, and large pieces of debris were removed by sieving the sediment over a coarse sieve (3 mm). Ten grams of sediment was mixed with Na_2SO_4, dried in an oven overnight at 40°C, and extracted with hexane:acetone (1:1) in a soxhlet for 16 hr. After cooling down, the extract was washed with 50 ml of deionized water and 5 ml of saturated NaCl solution, and the hexane fraction was concentrated to 3–4 ml by evaporation. Sulfur was removed using tetrabutylammonium sulfite (TBA) (De Voogt *et al.*, 1990; Verbrugge *et al.*, 1991). Further cleanup was performed using a multilayer acid–base silica column consisting of 0.75 g Na_2SO_4 on top of dried silica with, respectively, 0.75 g of 22% and 0.75 g of 40% hexane-washed H_2SO_4 and 1 g 33% NaOH on glass wool. After preeluting with 5 ml of hexane the column was loaded with the sample and eluted with 20 ml of hexane followed by 20 ml of hexane:dichloromethane (1:1). The sample was reduced to less than 1 ml by rotoevaporation at 35°C and transferred to 1-ml vials. Just before the extract was completely dried under a gentle, filtered air flow, 100 μl of DMSO was added and the last hexane was evaporated.

Collection and extraction of pore water. Pore water was collected by the method described by Maas *et al.* (1993). Mixed, decanted, and sieved sediment samples of 200 g were added to 500-ml plastic containers and centrifuged for 30 min at 3000*g* at 5°C. The supernatant was carefully decanted into glass erlenmeyer flasks and stored at 5°C. To avoid major chemical or physical changes in its composition, the pore water was not kept longer than 1 week. Samples of pore water (5 ml) were extracted with 5 ml hexane, vigorously vortexed, and centrifuged at 3000*g* for 5 min. The hexane fraction was collected in a hexane-rinsed, glass test tube. This procedure was repeated twice, and the combined hexane fractions were evaporated under a gentle nitrogen flow until only a very small drop was left. To each sample 200 μl of DMSO was added and, after complete evaporation of the remaining hexane, diluted with 3.3 times more DMSO.

Early Life Stage Test with Zebra Fish

Early life stage (ELS) tests with zebra fish (*B. rerio*) were performed according to Van Leeuwen *et al* (1990). Two to six females and two to four males were placed together overnight in special "brood chambers." Induced by the morning light, the females normally lay up to 600 eggs which are fertilized by the males. The fertilized eggs fall through a mesh at the bottom of the brood chamber into a separate compartment. After disinfection (in a 0.04% formalin solution for 1 min) 20 blastular eggs were transferred to 60-ml glass vials, each containing 20 ml of test solution. Undiluted pore water samples and two dilution steps of 1.8 and 3.1 times in Dutch standard water (DSW, pH 7.8, hardness = 210 mg/liter as $CaCO_3$) were prepared in triplicate, added to the eggs, and renewed every 2 to 3 days. DSW was used as a blank A TCDD standard series was included at the concentrations of 0, 25, 50, 500, 1000, and 2000 pM, added in 80 μl DMSO/20 ml DSW and renewed daily. During solution renewal, eggs or larvae were left in the test vessels in a small amount of fluid, while the water was changed. The embryo–larval stages were exposed continuously for 8 days at 25 ± 1°C, without feeding. Water quality parameters (pH, O_2, ammonium, nitrate, and nitrite) were checked throughout the test period and remained within acceptable ranges. Dead larvae were enumerated and removed daily, and malformations were recorded. At the end of the test period surviving fish were checked for malformations under a binocular microscope. The end points, percentage mortality and teratogenicity, were corrected by deducting the percentage dead in the control, multiplying the result by 100, and dividing this by 100 minus the percentage mortality in the control (Tattersfield and Morris, 1924).

Calculations of CALUX-TEFs and CALUX-TEQs

For calculation of the TCDD equivalency factors for compounds in the CALUX assay (CALUX-TEFs) a complete dose–response curve in triplicate was used. Values between 10 and 90% of the maximum were used for Scatchard analysis. The intercept with the *X*-axis yields the theoretical maximum CALUX response ($CALUX_{max}$), the intercept with the *Y*-axis the

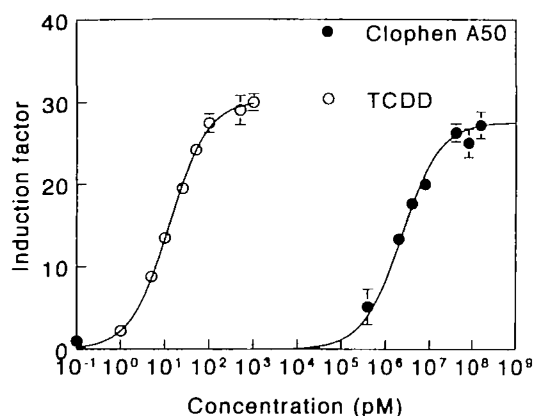

FIG. 1. Dose–response curves for luciferase induction in H4IIE.Luc cells for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the technical PCB mixture Clophen A50. Background CALUX response was 13.8 relative light units (RLU)/μg protein, induction by 1000 pM TCDD 414.2 RLU/μg protein. Exposure was during 24 hr, for details see Materials and Methods

$CALUX_{max}$/EC50 from which the EC50 can be calculated. The calculated EC50 was always compared with the visually determined values for confirmation. The CALUX-TEF value of each compound was calculated as the ratio EC50 (TCDD):EC50 (compound).

For calculation of TCDD-equivalents in the CALUX assay (CALUX-TEQs), a complete standard curve of 2,3,7,8-TCDD was used for each cell line. For each 24-well plate the response of the unknown samples were measured (in triplicate) plus three TCDD calibration standards bracketing the TCDD EC50. Based on these calibration standards the complete standard curve was adjusted for plate to plate variation. The standard curve was fitted (1-site ligand fit, function: $y = a_0 * x(a_1 + x)$) using SlideWrite 5.1, which determines the fitting coefficients by an iterative process minimizing the χ^2 merit function (least squares criterion). The CALUX-TEQ value for the unknown sample was interpolated on this curve. The response of the unknown sample is ideally in the range of 10–100% of the EC50 (for H4IIE.Luc 1–10 pM TCDD), and should be further diluted when out of this range. The detection limit is set at the DMSO response plus three times the standard deviation.

RESULTS

Induction of CALUX Response by PHAHs

The CALUX response was sensitive and reproducible for exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD). The detection limit in H4IIE.Luc cells was less than 1 pM, which equals an absolute amount of less than 0.5 fmol/well. The EC50 is reached at 10 pM and the curve saturates between 100 pM and 1 nM, with a maximum induction factor of 30 (Fig. 1). The standard deviation in the CALUX assay, performed in triplicate, was generally ≤5%. The dose–response curve for the technical PCB mixture Clophen A50 was comparable to that of TCDD, but the EC50 was 5×10^5 times greater and the maximum induction factor was 27, slightly less than for TCDD. Dose–response curves for mixtures of polychlorinated terphenyls (PCT) (Aroclor 5442 and 5460), polybrominated diphenylethers (PBDE), and individual PBDE congeners were similar (data not shown). The EC50 values, TEFs, and induction factors at the EC50 for CALUX activity in H4IIE.Luc cells exposed to these PHAHs are reported in Table 1. The CALUX-TEF for the technical PCB mixture Clophen A50 and for the PCT mixture Aroclor 5442 were almost identical. Aroclor 5460 was found to be 350 times more potent as a CALUX inducer, and the PBDEs tested were similar to Clophen A50. The CALUX-TEF of 2,2′,4,4′-TBDE was 10 fold less than for the other PBDEs, comparable to the relatively low induction potency of Ugilec 141. The TEFs for CALUX activity determined in H4IIE.Luc or Hepa.Luc cells were almost identical (data not shown).

The CALUX and EROD assay gave similar curves (Fig. 2), though in the CALUX assay the lower detection limit was approximately two- to threefold less and the maximum induction factor was approximately threefold higher, the EC50 values were somewhat less, and the TEFs slightly higher (both in H4IIE cells). The EC50 for TCDD was 16 pM in the EROD assay. The induction factors in the EROD assay for Aroclor 5442 and Aroclor 5460 at the EC50 were 4.0 and 3.6, respectively (data not shown), as compared to 14.0 and 8.5, respectively, in the CALUX assay. EROD induction by Ugilec was not measurable, and EROD induction by PBDEs was not studied.

Substrate Inhibition Tests

Inhibition of the responses of the CALUX and EROD assays by PCBs were compared in the H4IIE cells (Fig. 3a). The activity induced by 50 pM of TCDD was measured in the presence of increasing amounts of Clophen A50 added to the lysed cells shortly before the substrates luciferin or ethoxyresorufin, respectively, were added. The induction by 50 pM of TCDD for EROD activity was 9.6 pmol resorufin/μg protein·min, while that for CALUX activity was 98.9 relative light units (RLU)/μg protein. Both responses were set at 100%. The measured EROD activity was reduced to 16% of the original activity after addition of 100 nM Clophen A50. A Clophen A50 concentration of 50 μM completely eliminated EROD activity. No significant inhibition of the CALUX activity was observed at concentrations of Clophen A50 as great as 500 μM. Also 3,4,3′,4′-tetrachlorobiphenyl (PCB-77) inhibited EROD activity when added to the lysed cells just before measuring (Fig. 3b). This inhibition was, however, less pronounced than for Clophen A50, as 40% of the EROD activity was still measurable when 50 μM PCB-77 was added. Again, no significant inhibition was observed for CALUX activity (Fig. 3b). Comparable results were obtained using Hepa cells (data not shown), although the reduction in measurable EROD activity was slightly less than for H4IIE cells. No reduction of the CALUX activity was observed in either H4IIE.Luc or Hepa.Luc cells.

TABLE 1
Toxic Equivalency Factors (TEFs) for Luciferase Induction in H4IIE.Luc Cells of Several Sediment Associated Compounds, as Determined in the CALUX Assay

Compound	Molecular weight	EC50 (M)	CALUX TEF	Induction (fold)[a]
2,3,7,8-TCDD	322	1.0×10^{-11}	1 0	15.0
Clophen A50	300	$5 0 \times 10^{-6}$	$2 0 \times 10^{-6}$	13.6
Aroclor 5442	396	$6 3 \times 10^{-6}$	$1 6 \times 10^{-6}$	14.0
Aroclor 5460	562	1.8×10^{-8}	5.6×10^{-4}	8.5
Bromkal 70-5-DE	536	2.1×10^{-6}	4.8×10^{-6}	7.3
2,2′,4,4′-TBDE	486	1.4×10^{-5}	7.1×10^{-7}	7 6
2,2′,4,4′,5-PBDE	565	1.7×10^{-6}	5.9×10^{-6}	4.8
2,2′,4,4′,5,5′-HBDE	644	2.3×10^{-6}	4.3×10^{-6}	9.6
Ugilec 141	320	1.6×10^{-5}	6.3×10^{-7}	4.0

Note. The 50% effect concentrations were calculated using Scatchard analysis. TCDD, tetrachlorodibenzo-*p*-dioxin; TBDE, tetrabromodiphenylether; PBDE, pentabromodiphenylether; HBDE, hexabromodiphenylether.

[a] The induction factor is determined at the EC50, as the increase in luciferase induction relative to background induction (12.1–13.8 relative light units/μg protein).

Induction of CALUX Activity by Sediment Extracts

As an example of the type of responses observed, the dose–response curves for H4IIE cells exposed to sediment extracts from the relatively clean Oostvaardersplassen (OVP) and the polluted Ketelmeer (KM1) are presented for the CALUX assay (Fig. 4a) and the EROD assay (Fig. 4b). In the CALUX assay, the maximum induction factor for TCDD (1000 pM) was 44.6. The extract of 0.3 mg of KM1 sediment resulted in a 4-fold induction of CALUX. The extract of 193 mg of KM1 sediment resulted in a CALUX response comparable to 1000 pM TCDD. The induction by 387 mg of sediment was approximately 70-fold greater than the DMSO blank, which was greater than the TCDD maximum induction factor. The extract of 441 mg of OVP sediment did not induce CALUX activity more than 12.4-fold. The EROD activity was completely inhibited in the presence of KM1 extract at the greater concentrations. Based on the TCDD standards that were used in each CALUX assay, the TEQ value of the KM1 sediment was calculated to be 70.0 ± 6.6 fmol/g dry sediment and 4.2 ± 0.2 fmol/g for the OVP sediment. The recovery of ^{13}C-labeled PCBs and ^{14}C-labeled TCDD in the whole extraction and purification procedure was 85%. Because it was not possible to measure the recovery of each individual sample, the recovery was not used in the calculation of TEQ values.

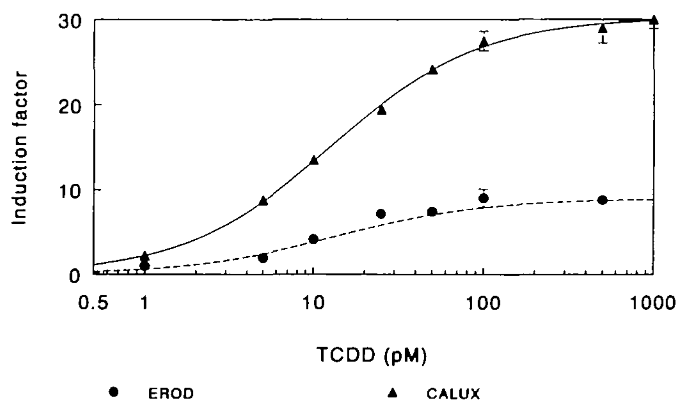

FIG. 2. Dose–response curves for luciferase induction in H4IIE.Luc cells and ethoxyresorufin *O*-deethylase (EROD) activity in H4IIE wild type cells for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). For conditions of the luciferase response, see Fig. 1 Background EROD activity was 1.3 pmol resorufin (RR)/μg protein · min, induction by 1000 pM TCDD 11.7 pmol RR/μg protein · min. Exposure for EROD assay was during 48 hr; for details see Materials and Methods.

The sediment extracts not subjected to clean-up, had a dark tar-like appearance (especially the extract of KM1) and caused visible disturbances in the cells, resulting in decreasing protein concentrations and finally cell death. Therefore, unpurified sediment extracts were not tested in further experiments.

CALUX Activity by Pore Water Extracts

In contrast to sediment extracts, unpurified pore water extracts did not result in any cell death or reduced protein concentrations. The dose–response curves in H4IIE.Luc cells of pore water extracts from OVP, KM13, and the North Sea Canal (NSC) are presented in Fig. 5. The extract of 250 μl NSC pore water induced CALUX activity 12.1-fold, but did not result in maximum induction. The same volume resulted in 3.8- and 2.2-fold induction for KM13 and OVP, respectively. When tested in the EROD assay, the same extracts resulted in 3- to 4-fold less induction (data not shown). Based on the measured TCDD standards, the TEQs calculated for these pore waters were 26.4 fmol/ml for KM13, 317.4 fmol/ml for NSC, and below detection limit for OVP. The detection limit in this CA-

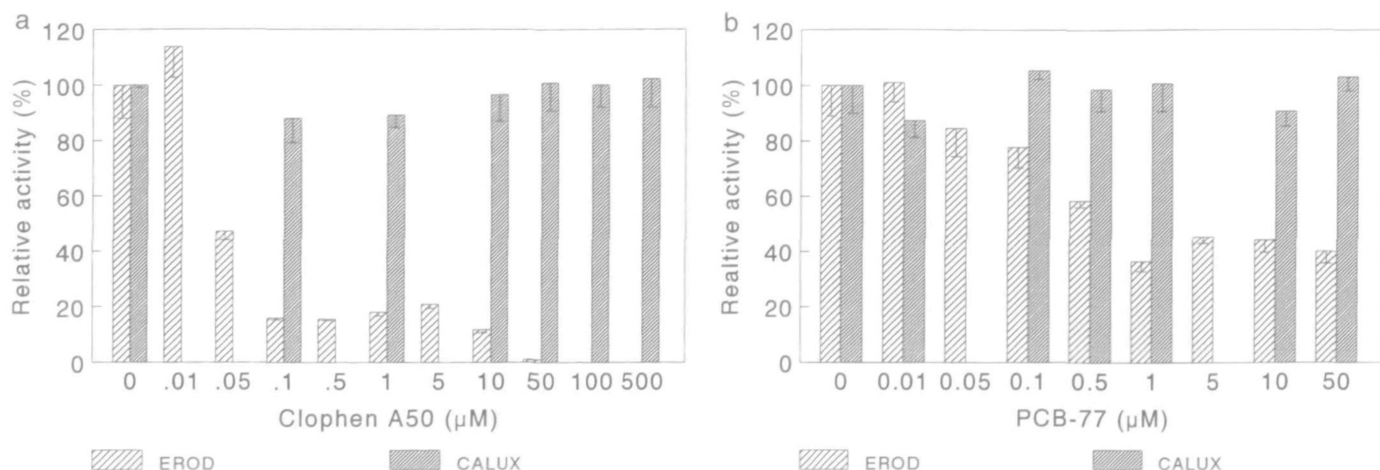

FIG. 3. Effect of an increasing concentration of (a) Clophen A50 or (b) PCB-77, added to lysed H4IIE.Luc or H4IIE.wt cells shortly before measuring, on measurement of, respectively, the CALUX or EROD activity. The CALUX and EROD activities had previously been induced by exposure to 50 pM 2,3,7,8-TCDD during 24 hr, and this induction was set at 100%.

LUX assay was 1.9 fmol/well. As 250 μl pore water or less was tested per well, the detection limit was 7.6 fmol/ml pore water. The recovery of ^{13}C-labeled PCB-101 (2,2',4,5,5-pentachlorobiphenyl) and PCB-153 in the extraction procedure was 94%, but the TEQ values were calculated without correction for the average recovery.

Early Life Stage Test and CALUX Assay with Pore Water

The slope of the dose–response curve for the ELS test with zebra fish in artificial pore water (DSW) spiked with TCDD was steeper than the slope of the dose–response curve for the CALUX assay (Fig. 6). The EC50 (8 days, malformations and mortality) for the ELS assay was 21 ± 2.3 pM TCDD (21 fmol TEQ/ml), which was in the same order of magnitude as the EC50 of 10 pM TCDD for the CALUX assay. At concentrations greater than 35 pM 100% effect was observed, and at concentrations greater than 165 pM all zebrafish larvae were dead within 8 days. Even at the lowest concentration tested (8.3 pM TCDD) 7% malformations were observed.

The results of the CALUX assay were compared with those of the ELS test, with pore waters from 10 natural sediments (Table 2). The effects varied among pore waters, ranging from 0 to 100% effect in the ELS assay and from detection limit to 317 fmol TEQ/ml pore water in the CALUX assay.

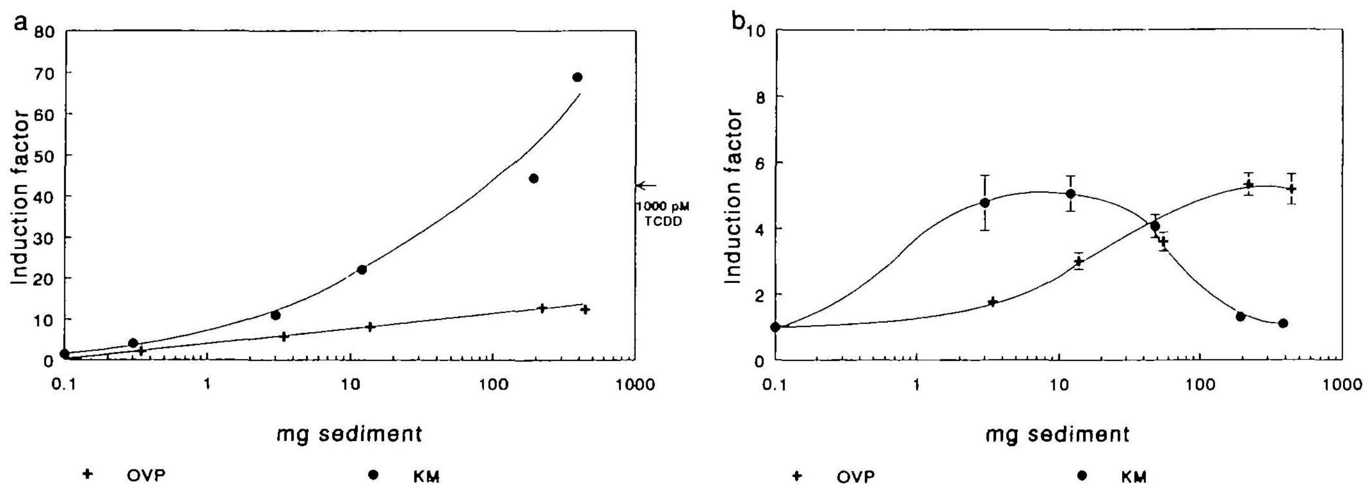

FIG. 4. (a) The CALUX response by extracts of Ketelmeer location 1 (KM) and Oostvaardersplassen (OVP) sediment in H4IIE.Luc cells after 20 hr of exposure, expressed as induction factor relative to background luciferase induction (12.1 relative light units/μg protein). (b) The same for EROD response in H4IIE wild type cells after 48 hr exposure, background EROD activity 1.1 pmol resorufin/μg protein · min.

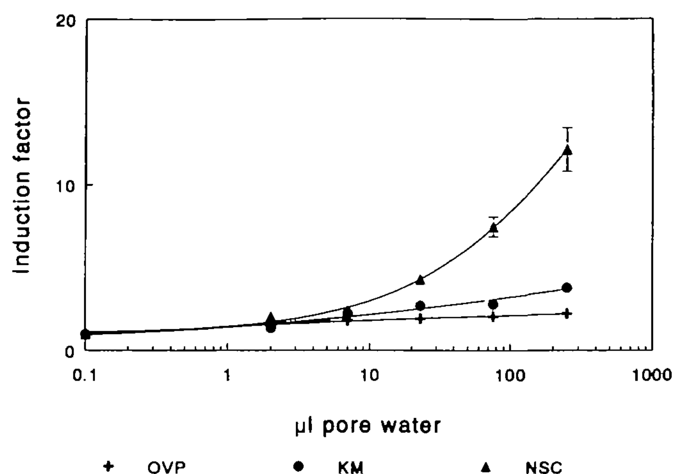

FIG. 5. The CALUX response by pore water extracts in H4IIE.Luc cells after 20 hr exposure, expressed as induction factor relative to background luciferase induction (12.1 relative light units/µg protein). OVP, Oostvaardersplassen; NSC, North Sea Canal; KM, Ketelmeer location 13.

DISCUSSION

The results demonstrate that the CALUX assay is a rapid, sensitive, and reproducible method for determining the toxic potency of mixtures of lipophilic compounds that bind to and activate the AhR. Persistent AhR-active compounds that occur in whole sediments or pore water were measured in small aliquots with minimal effort. The CALUX assay represents significant improvement relative to the H4IIE.wt assay, which uses endogenous EROD induction as a response. The sensitivity and linear working range of the CALUX was slightly better than the EROD assay, but the primary improvement of the CALUX assay was that it was insensitive to substrate inhibition, which has been a problem in the EROD assay.

Induction of CALUX Response by PHAHs

The TEFs of Clophen A50, Bromkal 70, PBDE, and HBDE (Table 1), were almost identical, which is in accordance with the results of an *in vivo* experiment with Wistar rats (Von Meyerinck *et al.*, 1990) in which the EROD induction by Bromkal 70 was slightly greater than the induction by Aroclor 1254, a technical PCB mixture comparable to Clophen A50 (Schultz, 1989). The TEF for coplanar and mono-*ortho*-polychlorinated diphenyl ethers (PCDE), based on immunotoxicity and AHH induction in C57BL/6 mice (Safe, 1990), was 10^{-3}. This is about 200-fold greater than the CALUX-TEFs that we determined for the di-*ortho*-brominated diphenylethers and the mixture Bromkal 70-5-DE. However, TEFs of di-*ortho*- and mono- or non-*ortho*-PCBs have been reported to differ by 50–200 as well (Safe, 1990). In the CALUX assay, the PCT Aroclor 5460 was 20-fold more potent than Aroclor 5442. A comparison of the EROD induction in Sprague–Dawley rats by PCT mixtures Aroclor 5432 and Aroclor 5460 (Toftgard *et al.*, 1986) revealed that Aroclor 5432 was, on a mass base, a more potent inducer than Aroclor 5460. Since Aroclor 5460 is much heavier than Aroclor 5432 or 5442 (Table 1) this comparison would be different on a molar base. We did not test Aroclor 5432. The CALUX-TEF for Ugilec 141 was 3.2-fold less than that for Clophen A50. This is in accordance with an earlier observation that the EROD activity in C57BL/6 mice dosed with 200 mg Ugilec 141/kg was 3.4-fold less than that of mice dosed with 200 mg Aroclor 1254/kg (Murk *et al.*, 1991). The molar densities of Ugilec 141 and Aroclor 1254 differ less than 10%.

It should be noted that induction values reported for technical mixtures could be partially due to a small percentage of impurities. Additionally, it is important for TEF calculations that the concentrations of the stock solutions used for the assays are validated with GC-MS, as concentrations of carefully prepared stock solutions from different laboratories may vary by a factor of 10. If no validation has been performed, the EC50 of the TCDD stock used should be mentioned as well. The maximum CALUX induction factors for TCDD may differ slightly between individual assays. This is probably due to slight differences in the condition of the cells, such as the degree of confluency. Three TCDD calibration concentrations were measured in duplicate on each 24-well plate to allow comparison of TEFs and TEQs determined in different assays.

Substrate Inhibition in the EROD Assay

The CALUX assay offers some advantages when compared to the commonly used EROD assay in H4IIE cells. It is

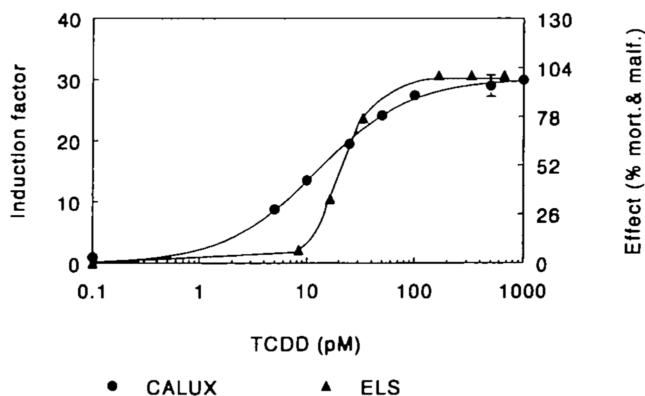

FIG. 6. Dose–response curves for mortality and malformations after 8 days exposure of eggs of zebrafish (*Branchycardio rerio*) (ELS response) and luciferase production (CALUX response) in H4IIE.Luc cells after 24 hr exposure, compared for the same 2,3,7,8-TCDD concentrations. The CALUX response is expressed as induction factor relative to background induction (13.1 relative light units/µg protein). The value at 0.1 pM is in fact the control value (0 pM).

TABLE 2
Concentrations of Several Contaminants, Sediment Quality Classification, and Toxicity of Pore Water Samples

Location (abbreviations in Materials and Methods)	CD (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Σ10 PAHs (mg/kg)	PCB-153[a] (μg/kg)	Classification[b]	CALUX-TEQ (fmol/ml)	ELS (% effect)
KM3	5.97	98.63	873.88	11.43	27.97	4	24.0	42
KM13	12.52	134.44	1333.6	5.0	41.43	4	26.4	100
OVP	1.10	21.64	214.44	0.83	2.08	1	dl[c]	4
BB	5.02	48.59	679.85	10.57	25.78	3	28.4	100
DOM	39.94	311.77	1269.3	6.87	30.36	4	17.6	100
DL	0.73	19.84	126.25	0.36	dl[d]	1	dl[c]	0
SB	7.72	75.40	859.26	7.37	59.32	4	30.8	100
MM	0.75	19.09	137.76	0.81	dl[d]	0	dl[c]	10
DM	0.52	14.20	102.51	5.59	dl[d]	2	dl[c]	0
NSC	2.27	77.32	340.92	25.52	86.21	3	317.4	100

Note. TCDD equivalents were determined in the CALUX assay (CALUX-TEQs); toxicity is expressed as the response in the early life stage (ELS) test with zebrafish (*Branchycardio rerio*) exposed to 10 undiluted pore waters. Concentrations of 3 heavy metals (Cd, Cu, Zn), 10 poly aromatic hydrocarbons (PAHs), and PCB-153 in sediment samples from the same locations (unpublished data RIZA) The sediments were classified relative to sediment quality standards, calculated as standardized sediment (Derde Nota Waterhuishouding, 1993).

[a] PCB-153, 2,2′,4,4′,5,5-hexachlorobiphenyl.

[b] Class 1 meets General Environmental Quality Criteria 2000; Class 2 meets testing value (unpolluted, though further research may be necessary); Class 3 meets signal value (clean-up may be required following further research); Class 4 exceeds signal value (sanitation required) (CCRX, 1990).

[c] Below detection limit CALUX assay (7.6 fmol/ml in this experimental setup; see also Results: Pore water measurements).

[d] Below detection limit GC-MS (1 μg/kg).

slightly more sensitive and has a threefold greater induction factor, but, more importantly, the CALUX assay offers the possibility of measuring the presence of less potent inducers such as Clophen A50 and Ugilec 141, as well as low concentrations of compounds in environmental matrices. Since PHAHs are not substrates for luciferase, substrate inhibition by PHAHs will not occur in the CALUX assay. The EROD activity is already inhibited at substrate concentrations of 50 nM or more, which are needed for induction by less potent inducers (Fig. 3a). In contrast to Clophen A50, PCB-77 did not inhibit EROD activity up to 99% at high concentrations (Fig. 3b). Since PCB-77 is a substrate for cytochrome P4501A1, and readily metabolized in *in vitro* incubation with rat hepatic microsomes (Murk *et al.*, 1994), PCB-77 could have been metabolized for 40% or more during 60 min incubation in the H4IIE.wt EROD assay. Substrate inhibition has been demonstrated in chicken hepatocytes and rat and fish hepatoma cell lines (Kennedy *et al.*, 1993; Sawyer *et al.*, 1984; Hahn *et al.*, 1993; Richter *et al.*, 1996). Also in experimentally dosed flounder the EROD activity induced by 5 μg TCDD/kg was reduced by 66% when dosed simultaneously with 42 mg Clophen A50/kg, although the cytochrome P450 protein content increased in an additive manner (Besselink, pers. comm.).

CALUX Activity Induced by Sediment and Pore Water Extracts

Exposure of H4IIE.Luc cells to extracts of KM1 sediment, which is known to contain a number of contaminants, resulted in 17-fold greater CALUX activity than did extracts from the relatively clean OVP sediment. Based on chemical analyses, the TEQs of these two locations differ by a factor 20 (unpublished data RIZA). A comparable difference was observed using pore water extracts. The CALUX response to 250 μl OVP pore water was less than the detection limit (1.9 fmol/250 μl). The NSC pore water sample contained almost 13-fold more CALUX-TEQs than the two sites from the KM. A herbicide producing plant is situated in the NSC, where accidental emissions of PCDD and PCDF have occurred (Turkstra and Pols, 1989). In contrary to whole sediment samples, pore water needed no soxhlet extraction or clean-up steps. This makes the sample preparation much more rapid and the chance of loosing unknown AhR-active compounds with yet unknown optimal recovery conditions much smaller.

PHAHs and organochlorine pesticides can be lost during filtration, due to irreversible adsorption on polyamide filters, and to a lesser degree, on Teflon material (Rood *et al.*, 1995a,b). This may explain why Anderson *et al.* (1995) did not measure any CALUX activity using pore water samples from polluted sites, which they filter sterilized over a 0.1-μm membrane. Additionally, Anderson *et al.* (1995) kept collected pore water for approximately 4 months before testing. Whole sediments can be kept at 4°C for more than 112 days without losing toxicity (Othoudt *et al.*, 1991); however, pore water samples should not be kept longer than 1 week because of chemical changes (Burton, 1991;

Hill *et al.*, 1994). When optimizing the pore water assay, we observed that upon filtering [^{3}H]PCB-77-spiked pore water samples over a glass fiber Whatman GF/F 0.7-μm filter, 86–92% of all radioactivity remained on the filter. This was to be expected, since lipophilic molecules like PHAHs are mainly associated with dissolved and particulate organic carbon, especially in the smallest size fraction (0.22–1 μm) (Muir *et al.*, 1992). PHAHs that are associated with small particles can readily be accumulated by species which filter or ingest organic particles, like mussels and chironomids (Muir *et al.*, 1992; Ankley *et al.*, 1992), and will be relatively available for uptake through skin or gills after dissociating into the water phase (Swartz *et al.*, 1990; Kjeller *et al.*, 1990; di Toro *et al.*, 1991; Power and Chapman, 1992). Partitioning of organic compounds between the solid phase of sediments and pore water depends on the lipophylicity of the compounds and the presence of dissolved organic matter like humic acids in the pore water (Landrum and Robbins, 1990). Analysis of only the solid phase of sediments does not discriminate between bioavailable and tightly bound contaminants. Therefore, either much additional information is needed for assessment of the bioavailable fraction of sediment associated contaminants or the biologically available fraction could be measured directly using pore water samples.

In Vivo Validation of the CALUX Response

The CALUX response has already been chemically validated with pure compounds (Aarts *et al.*, 1995; Garrison *et al.*, 1996) or with mixtures (Postlind *et al.*, 1993). To indicate the hazard of a certain polluted sediment for the ecosystem, the CALUX response has to be validated against responses from *in vivo* assays. In this study the *in vivo* response in the ELS assay for a TCDD-spiked DSW sample corresponded with the CALUX response (Fig. 6), although the slope of the ELS assay curve was steeper, resulting in a more narrow working range. These results demonstrate that compounds present in pore water samples are directly available for organisms in the water phase, since the fish larvae did not eat during the test period. The response in the 8-day ELS assay with zebra fish has been reported to correlate with the 60-day ELS test for rainbow trout and with a chicken embryotoxicity tests (Van Leeuwen *et al.*, 1990). These species share embryonic development in the absence of maternal metabolism or a placenta barrier. Although the cells used in the CALUX assay are mammalian, they are also directly exposed to the toxic compound. The correlation with *in vivo* embryotoxicity in mammals could therefore be less strong.

No clear correlation was observed when comparing the CALUX response to extracts of naturally occurring pore waters with the response in the ELS test (Table 2). This was to be expected, since the ELS assay will also be influenced by the sometimes great concentrations of heavy metals present in the whole pore water samples (Table 2). A better correlation with the CALUX response is to be expected with an ELS assay performed with extracts from pore waters. Comparison of the concentrations of PCB-153 in the sediments with the CALUX response shows a better correlation (Fig. 7).

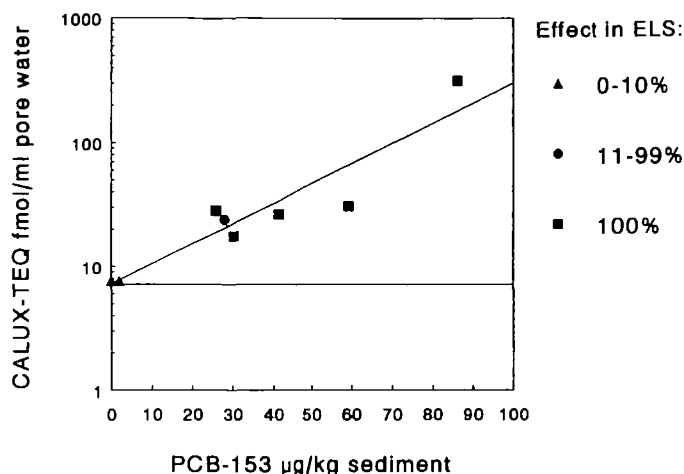

FIG. 7. Correlation between the 2,3,7,8-TCDD equivalents in pore water determined in the CALUX assay (CALUX-TEQs) and the PCB-153 level in sediment from the same location measured by GCMS (Table 2). The different symbols indicate the percentage mortality and malformations in the early life stage test with zebrafish (*Branchycardio rerio*) performed in the collected pore water (see also Table 2).

The Use of the CALUX Assay for Hazard Assessment of Sediments

Given the complexity of the mixtures of PHAHs in sediments and organisms in industrialised countries, chemical analysis can give only a rough impression of the potential risks for the environment. Due to the often small concentrations of individual congeners and the presence of unknown or not routinely measured AhR active substances, like PBDEs and PCTs that may still contribute to the total TEQs of a sample, there is a risk of underestimation of the total TEQ. The toxic responses of AhR-active compounds may be additive, as is the case for TCDDs and TCDFs, but for PCBs and non-PCB-like substances both additive and antagonistic interactions have been observed (Safe, 1994; Aarts *et al.*, 1995). These limitations form drawbacks to the TEQ approach. The CALUX assay provides a measure of the toxic potency of the whole mixture, including interactions. To be able to correct for differences in quantification due assay to assay variation, three TCDD calibration standards have to be measured with each assay (Murk *et al.*, 1996). No changes in EC50 values or CALUX induction relative to TCDD have occurred over the period of 1 year that the cells were in culture.

For assessment of the total biological hazard by contami-

nants in sediments, a triad approach of combined chemical analyses, bioassays, and *in situ* bottom fauna studies can provide an integrated diagnosis of sediment contamination (Chapman, 1992; Van de Guchte, 1992, 1995). *In vivo* bioassays will give an impression of the total toxic potential and the bioavailability of sediment-associated contaminants. However, they are time-consuming, vulnerable to physical or chemical conditions of the samples, and often provide little information on the cause of toxicity. Additional *in vitro* bioassays like the CALUX assay will provide more specific insight into the functional groups of chemicals that are present. In particular the CALUX assay using pore water extracts could be a useful tool for rapid and sensitive indication of the toxic potency of biologically available mixtures of AhR-active compounds in sediments. If high CALUX responses are observed, chemical analysis is needed to determine the specific compounds responsible for the toxic response.

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