Application of the *CALUX* (chemical activated luciferase gene expression) assay for measuring TCDD-equivalents in sediment, pore water and blood plasma samples

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1. Abstract

The results presented in this paper demonstrate that the *in vitro CALUX* (chemical activated luciferase gene expression) bioassay is a rapid, sensitive assay for assessing the toxic potency of (mixtures of) Ah-receptor active compounds in sediment, pore water and blood plasma. Recombinant rat (H4IIE) and mouse (Hepa1c1c7) hepatoma cell lines, containing arylhydrocarbon receptor (AhR)-mediated luciferase gene expression, were used to determine the *CALUX*-response of sample extracts. Using a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard curve, the *CALUX* responses for the samples were converted into so-called *CALUX*-TEQs (TCDD-equivalents). The detection limit of the *CALUX* assay was 0.5 fmol of TCDD-equivalents. The *CALUX* activity induced by organic extracts from 450 mg aliquots of sediment or 250 μl aliquots of pore-water corresponded well with the instrumentally analysed level of dioxin-like compounds in the sediment. Using pore water, only a simple and rapid extraction procedure was needed, without additional clean-up to prevent cell death. The *CALUX* response both for the fatty, uncleaned organic extract and the cleaned extract of blood plasma from experimentally exposed eider ducks, correlated significantly with the PCB-levels measured in abdominal fat or blood plasma, and with the hepatic EROD activity in the same animals.

An important advantage of the *CALUX*-assay compared to the ethoxyresorufin *O*-deethylase (EROD) assay is that the *CALUX* assay is insensitive to substrate inhibition, which is demonstrated in this paper for 3,3',4,4',5-pentachlorobiphenyl (PCB-126).

2. Introduction

For hazard and risk assessment of mixtures of PHAHs the concentrations of individual PHAHs multiplied by their respective toxic equivalency factors (TEFs) are added up to give the total TCDD toxic equivalency of the mixture[1]. Given the complexity of the mixtures of PHAHs in sediments and organisms, chemical analysis can only give a rough impression of the potential health risks. Due to the often small concentrations of individual congeners and the presence of unknown or not routinely measured AhR active substances, 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 polychlorinated dibenzo -p-dioxins (PCDDs) and -furans (PCDFs), but for PCBs and non-PCB like substances both additive and antagonistic interactions have been observed[2,3]. These limitations are drawbacks to the TEQ-approach. The *CALUX* bioassay provides a

measure of the toxic potency of the whole mixture, including interactions.
This paper presents some examples of the use of the *CALUX* assay for monitoring Ah-receptor active compounds in sediment, pore water and blood plasma. Applications of the *CALUX*-assay for human milk and blood samples, butter fat and coconut oil, otter liver and whole blood, and fish samples[4,5,6], and application of the *CALUX* assay for compounds that bind to and activate the estrogen receptor[7], will be presented separately at Dioxin 1996. In pursue of the substrate inhibition tests with Clophen A50 and PCB-77 reported elsewhere[8], possible substrate inhibition of the more potent Ah-receptor agonist PCB-126 is also determined, by comparing the dose-response curves in the EROD and *CALUX* assay.

3. Methods

Sample collection and preparation

Sediment samples differing in degree of pollution were obtained from the National Institute for Inland Water Management (RIZA)were decanted and sieved, 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[8]. Sulphur was removed using tetra butyl ammonium sulphite (TBA). Further clean-up was performed using a multi-layer acid-base silica column consisting of 0.75 g Na_2SO_4, on top of dried silica with 0.75 g of 22% and 0.75 g of 40% hexane washed H_2SO_4, and 1 g of 33% NaOH on glass wool. The column was eluted with 20 ml of hexane followed by 20 ml of hexane:dichloromethane (1:1), dried under a gentle, filtered air-flow, and dissolved in 100 µl of DMSO. Pore water was collected from 200 g decanted and sieved sediment by centrifugation for 30 min at 3000 g at 5°C. The supernatant was carefully decanted into glass erlenmeyer flasks and stored at 5°C. Samples of 5 ml pore water were extracted three times with 5 ml hexane, the hexane was evaporated under a gentle nitrogen flow, and the extract dissolved in 200 µl of DMSO. Blood plasma was collected from 27 days old eider ducklings, was collected on day 10 after i.p. injection with 5 ml corn oil/kg body weight (bw), 5 or 50 mg PCB-77/kg bw, or with 50 or 200 mg Clophen A50/ kg bw[10]. EROD activities were measured in hepatic microsomes, and PCB levels in abdominal fat (using GC-ECD)[11]. Blood plasma aliquots of about 1.5 ml were denaturated with an equal amount of methanol. The PHAHs were extracted three times with 3 ml of hexane. After the first extraction step, 3 drops of 6 M HCl were added to the water phase. Part of the extract was taken for further clean-up, the rest was evaporated at 30°C under a gentle stream of nitrogen gas, and dissolved in DMSO for use in the *CALUX* assay using Hepa.Luc cells. For the first dilution step these fatty extracts had to be kept warm (±30°C) to prevent clotting of the lipids in the pipet. The other part of the extract was cleaned over a 20% H_2SO_4 deactivated silica column with hexane-diethyl ether (97:3, v/v). This extract was evaporated, dissolved into isooctane, and analysed by GC. After GC-analysis, the remaining extract was evaporated and dissolved in DMSO for measurement of *CALUX*-activity in H4IIE.Luc cells.
For the ***CALUX-assay***, H4IIE.pGudluc1.1 (H4IIE.Luc) or Hepa1c1c7.pGudluc (Hepa.Luc) cells prepared as previously described[3,9] were exposed to PHAHs in 24-well culture plates[8]. Cells were seeded in 500 µl growth medium and incubated for 24 hr until the cell layer was 80-90% confluent. The medium was replaced by fresh growth medium containing the test compound in maximal 0.5% DMSO. After 24 hours incubation the cells were rinsed twice with 50% diluted PBS (0.5* PBS), 75 µl lysis mix was added, the cells were harvested 15 minutes later and centrifuged for 90 sec. at 13000 g. The supernatant was frozen at -80°C. For luciferase measurement 20 µl supernatant was pipetted in a 96-well microtiter plate, 100 µl luciferin assay mix (Promega) was added and after 90 sec mixing on a plate mixer the light production was measured in an Amerlite Luminometer. For calculation of *CALUX*-TEQs a standard curve of TCDD was fitted, and the *CALUX*-TEQ value for the unknown sample was interpolated on this curve[8]. For the substrate-inhibition test the *CALUX* assay has been performed in 96-well culture plates. Briefly, H4IIE.Luc cells were seeded in 96-well plates in 100 µl growth medium. After 24 hr incubation at 37°C the cell layer was 80-90% confluent, and 100 µl of fresh medium containing the test compound in maximal 0.5% DMSO was added to each well. After an additional 24 hr incubation, the plates were rinsed

twice with 0.5*PBS, 20 µl lysis buffer was added, and the plates were placed at -80°C. The plates were measured in a Labsystems Luminometer (Merlin) with automated injection. To be able to correct for differences in quantification due to assay to assay variation, three TCDD calibration standards were measured with each assay.

The ***EROD assay*** was mainly based on the method described before[12]. Briefly, H4IIE wild type cells were seeded in 96-well plates and exposed as described for the *CALUX* assay in 96 well plates, but exposure was during 48 hr instead of 24 hr. Instead of lysis mix, 20 µl of nanopure water was added before the plates were placed at -80°C. To measure resorufin production, the plates were pre-incubated for 20 min. at 37°C, with 50 µl of Tris-sucrose buffer (pH 8) with 40 µM dicumerol, and 25 µl 10 µM 7-ethoxyresorufin (ER) was added to each well. The reaction was started with 25 µl 1 mM NADPH per well, and after 1 hr incubation at 37°C, the resorufin production was measured in a fluorometer (Cytofluor) with excitation at 530 nm and emission at 590 nm.

For the *CALUX* or EROD ***Substrate inhibition assay***, H4IIE.Luc or H4IIE wild type cells were exposed to 50 pM TCDD in 96-well plates for 24 hr, and frozen at -80°C as described above. The plates were thawed on ice prior to luciferase or EROD activity measurement, and final concentrations of PCB-126 ranging from 1 to 10000 nM were reached by adding appropriate concentrations of stock solutions in 4 µl DMSO to each well. The plates were thoroughly mixed on a plate mixer before *CALUX* or EROD activity were measured as described above.

GC-ITD analysis was performed using the isooctane extract of eider duck blood plasma (see above). The gas chromatography (GC) column DB5-MS, 30 m x 0.2 µm (J&W Scientific) was coupled to a Saturn II ion trap detector (ITD; Varian). A retention gap of 2 m x 0.53 mm i.d. deactivated fused silica was used (Chrompack). The GC and ITD conditions are described elsewhere[13].

4. Results and discussion

The *CALUX* response was found to be very sensitive and reproducible using TCDD as a positive control. The detection limit in H4IIE.Luc cells was less than 1 pM, which equals an absolute amount of less than 0.5-0.2 fmol/well, depending on method of exposure used[8]. The EC_{50} was 10 pM, the TCDD dose-response curve saturated between 100 pM and 1 nM, and the standard deviation was generally ≤ 5%.

Exposure of H4IIE.Luc cells to extracts of Ketelmeer (KM) sediment, which is known to be polluted with several PHAHs, resulted in 17 fold greater *CALUX* activity than did extracts from the relatively clean Oostvaardersplassen (OVP) sediment[8]. Based on chemical analyses, the TEQs of these two locations

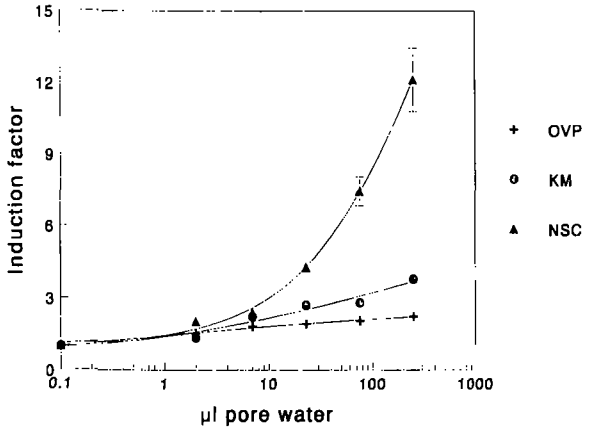

Figure 1 The *CALUX* response by pore water extracts in H4IIE.-Luc cells, expressed as induction factor relative to background induction. OVP= Oostvaarders-plassen, NSC= North Sea Canal, KM= Ketelmeer

differed by a factor 20. A comparable difference was observed using pore water extracts. Pore water from the North Sea Canal (NSC) contained almost 13 fold more *CALUX*-TEQs than the polluted KM (Figure 1). The high signal in the NSC was probably caused by accidental emissions of PCDD and PCDF from a herbicide producing plant situated in the NSC. Analysis of only the solid phase of sediments does not discriminate between bioavailable and tightly bound contaminants, where pore water samples represent the biologically available fraction of sediment contamination. An advantage of analysis of pore water is that no soxhlet extraction is needed, or clean-up to prevent cell-death. 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.

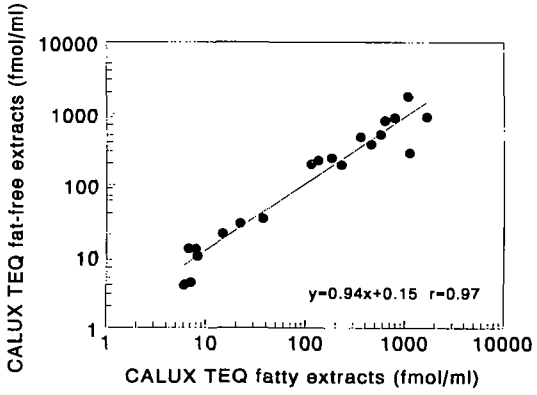

Figure 2 Correlation between *CALUX* TEQ of blood plasma extracts from experimentally exposed eider ducks, with and without clean-up.

Extracts of eider duck blood plasma's induced *CALUX* responses in a dose-related manner (Murk et al., submitted). The *CALUX*-TEQs based on the fatty blood plasma extracts correlated significantly with either the PCB-levels in the abdominal fat (r= 0.80-0.94, depending on the PCB-congener used), with the hepatic EROD activity of the eider ducks (r=0.88), and with the *CALUX*-TEQs determined for the cleaned extracts (Figure 2; r=0.96). Also the PCB levels in blood plasma extracts correlated well with the *CALUX*-TEQs measured in these cleaned extracts (r=0.86-0.96, depending on the PCB-congener used) (data not shown; Murk et al., submitted). These results demonstrate that blood plasma extracts can be measured in the *CALUX* assay with and without a clean-up procedure. Performing a simple Silica-H_2SO_4 clean-up, however, offers the possibility to concentrate the extract, and makes it easier to manipulate the extract. The observed good correlation of the *CALUX*-TEQ in blood plasma with the PCB-levels in abdominal fat suggests that the *CALUX*-TEQ in blood plasma provides an integrated measure of toxic potency of the internal dose of the AhR active PHAHs. Although the experimentally dosed eider ducks used for this study contained relatively high levels of PCBs, the *CALUX*-assay has already been applied for measuring TEQs in environmentally exposed species, such as cormorants, otters[6] and humans[4]. About 0.5-1 ml of blood plasma is needed for quantifying *CALUX*-TEQs in blood plasma, depending on the (expected) level of exposure (Murk et al., submitted).

Substrate inhibition

The *CALUX* assay offers some advantages when compared to the commonly used EROD assay in H4IIE cells. It is slightly more sensitive and has a three fold greater induction factor[8,12], but, more importantly, the use of the *CALUX* assay is not limited by substrate inhibition[8]. As is shown in Figure 3, the *CALUX* and EROD induction both increase with increasing PCB-126 concentration. The EROD activity, however, decreases again at concentrations greater than 100 nM, while the *CALUX* activity saturates at the maximum level (Figure 3). For the potent Ah-receptor agonist PCB-126, maximum induction levels are

not significantly different from the maximum induction with TCDD. However, the maximum EROD induction declines with decreasing AhR inducing potencies of PHAHs (Murk et al., in prep.). Figure 4 demonstrates the *CALUX* and EROD activity, induced by 50 pM of TCDD, when increasing amounts of PCB-126 are added to the lysed cells shortly before adding the respectively substrates. The measured EROD activity was already reduced to 73% of the original activity by addition of 10 pM of PCB-126, and after addition of 10 nM PCB-126 only 6% of the EROD activity is left. No inhibition of the *CALUX* activity was observed at any of the PCB-126 concentrations tested, which was to be expected, since PHAHs are not substrates for luciferase. These finding are in accordance with previously reported results[8] for H4IIE cell lines, and with results with experimentally dosed flounder, where the EROD activity induced by 5 μg TCDD/kg was strongly reduced when dosed simultaneously with Clophen A50/kg, although the cytochrome P450 protein content increased in an additive manner[14].

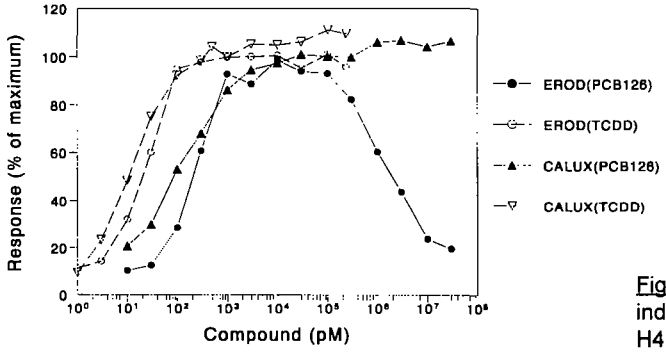

Figure 3 CALUX and EROD induction by PCB-126 in resp. H4IIE.Luc and H4IIE.wt cells. Exposure was during 24 hr in 96-well culture plates. Maximum induction was reached by 1000 pM of TCDD.

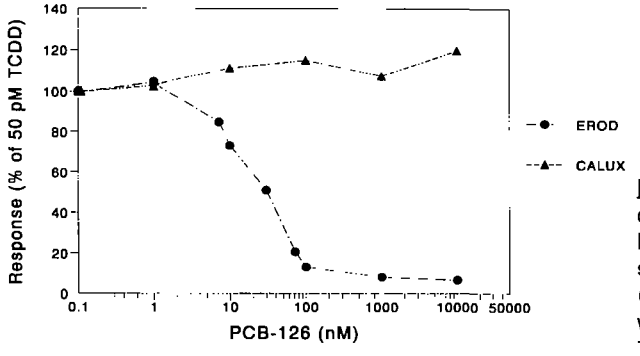

Figure 4 Effect of an increasing concentration of PCB-126 added to lysed H4IIE.Luc or H4IIE.wt cells shortly before measuring the *CALUX* or EROD activities, which were induced by exposure to 50 pM TCDD during 24 hr (this induction was set at 100%).

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