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Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*)

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Abstract

In the serious decline of European otters (*Lutra lutra*) over the last decades, polychlorinated biphenyls (PCBs) are considered to be one of the major factors. As no experiments can be conducted with otters, an eco-epidemiological study was performed to derive no observed effect concentrations (NOECs) for PCBs in the otter. A strong negative correlation was found between hepatic vitamin A and polychlorinated biphenyl (PCB) concentrations expressed as TCDD-equivalents (TEQs), coinciding with a higher incidence of infectious diseases. The no-effect concentration for vitamin A reduction was 2 ng TEQ/g lipid, 10-fold reduction was already found in animals with 5 ng TEQ/g lipid. The TEQ-levels measured with a reporter gene assay based on chemical-activated luciferase expression (the CALUX assay) correlated well with the TEQ levels calculated based on non- and mono-*ortho* PCB concentrations. The TEQ levels in blood and liver correlated well when expressed on a lipid basis. In living captive otters blood plasma TEQ levels (either measured based on gas chromatography (GC) or CALUX measurement) were lower than in the feral otters, and positively correlated with plasma total and free thyroid hormone but not with plasma retinol levels. Hepatic vitamin A concentration was found to be a physiologically relevant effect parameter. The NOEC for hepatic vitamin A reduction was translated into TEQ levels in fish and sediment. The CALUX response in 50–500 μl blood plasma proved to be a sensitive non-destructive biomarker for quantification of internal TEQ levels. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Vitamin A; Thyroid hormone; Blood; CALUX assay; PCB (polychlorinated biphenyl); TEQs (TCDD-equivalents)

1. Introduction

Over the last decades in Europe otter (*Lutra lutra*) populations have declined markedly. In addition to physical threats, such as habitat destruction, traffic accidents and drowning in fishing nets, also polyhalogenated hydrocarbon (PHAH) and, more specifically, polychlorinated biphenyl (PCB) pollution is considered to be one of the major factors in this decline. This assumption was based on toxicological studies with the mink (*Mustela vison*) which is often used as a model for the otter (Jensen et al., 1977; review in Leonards et al., 1994a), and on associations between high PCB concentrations in otters and declining or endangered populations (Olsson and Sandegren, 1983; Broekhuizen, 1989; Mason, 1989). For practical and ethical reasons no toxicological experiments have been conducted with the otter itself. The Dutch government aims at the return of the otter in The Netherlands (Van der Weiden et al.,

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1996), therefore knowledge about the environmentally safe concentrations of PCBs permitting survival of viable otter populations is needed. Because of the current lack of necessary data, a field study was performed to derive a 'no observed effect' level for the otter in the otter itself, its food and sediment. In addition, the possible use of the applied parameters as potential non-destructive biomarkers was studied, as such biomarkers will be needed to monitor exposure and health status of otters after re-introduction in their natural environment.

PCBs are known to exert their toxic effects via at least two mechanisms of action. Most of the toxic effects induced by PCBs, such as immunotoxicity and teratogenicity, are suggested to be mediated by the arylhydrocarbon receptor (AhR) (McConnell, 1985; Goldstein and Safe, 1989; McConnell, 1989; Safe, 1990; DeVito and Birnbaum, 1994). Based on these so-called 'dioxin-like' effects the TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) or toxic equivalency factor (TEF) concept was introduced (Safe, 1987) to be able to estimate the AhR-related toxic potency of a mixture of PCBs based on chemical data. For this purpose the concentrations of individual PHAHs are multiplied by their respective TEF value, and added up to give the total TCDD toxic equivalency of the mixture (Ahlborg et al., 1994). Recently a novel in vitro reporter gene assay has been developed to directly measure the AhR-related toxic potency of a mixture. This so-called CALUX (chemical-activated luciferase gene expression) assay is based on AhR-mediated firefly (*Photinus pyralis*) luciferase expression in genetically modified cell lines (Denison et al., 1993; Aarts et al., 1995; Garrison et al., 1996). A vector containing the luciferase gene under transcriptional control of DREs was stably transfected into a number of cell lines, including the rat (H4IIE) hepatoma cell line. In this CALUX cell line luciferase induction by TCDD is dose dependent, has a detection limit of less than 1 pM and saturates at ligand concentrations greater than 500 pM (Murk et al., 1996a). For the PCDD, PCDF and PCB congeners tested so far, the relative potency to induce CALUX activity correlated well with proposed TEF values (Aarts et al., 1995; Garrison et al., 1996; Sanderson et al., 1996). These TEF values are based on a number of endpoints, but the cytochrome IA-inducing potency in H4IIE hepatoma cells is most important (Ahlborg et al., 1994). The luciferase induction of an unknown sample is expressed as the so-called CALUX-TEQs, using a TCDD standard curve (Murk et al., 1996a, 1997).

Apart from the AhR-mediated toxicity, at least one additional mechanism of action is involved in PCB-induced toxicity. Hydroxy (OH^-) metabolites of PCBs have been shown to inhibit thyroxine (T4) binding to the T4 plasma transport protein (TTR) (Brouwer and Van den Berg, 1986; Lans et al., 1993, 1994). The T4–TTR complex is transported in the blood plasma in a complex with retinol bound to retinol-binding protein (RBP). Competition of OH-PCBs with T4 for TTR binding results in loss of T4 and of retinol–RBP from the body via the urine (Brouwer et al., 1989a). OH-PCBs have also been shown to exhibit other adverse effects, such as competitive inhibition of T4 deiodinase activity involved in the conversion of T4 to the active form of thyroid hormone, triiodothyronine (T3), and uncoupling of mitochondrial oxidative phosphorylation (Brouwer et al., 1994).

Many toxic symptoms of PCB-exposure resemble those of vitamin A deficiency (Brouwer, 1991; NORD, 1992), a vitamin which plays an important role in tissue development in fetuses, reproduction, and resistance against infectious diseases. As a consequence of both mechanisms of toxic action of PCBs mentioned above, vitamin A homeostasis will be disturbed and vitamin A storage in liver reduced, as has been demonstrated in several experimental and field studies (Jensen et al., 1987; Spear et al., 1988; Brouwer et al., 1989b; Spear et al., 1989; Brunström et al., 1991; Chen et al., 1992; Zile, 1992; Murk et al., 1994). Therefore, reduction in hepatic vitamin A concentrations is expected to be a sensitive, and physiologically relevant marker for the toxic action of PCBs.

A joint study has been performed with environmentally exposed feral and captive otters. This paper describes the comparison of hepatic retinoid concentrations, as a potential biomarker for effect, with the AhR-related toxic potency of PCBs in the same otter livers. In addition, these results were compared with information on the health status of the dead otters, which is presented in more detail by Leonards et al. (1997a).

For the animals from which liver as well as whole blood samples could be collected, gas chromatography (GC)- and CALUX-TEQ levels were determined in both matrices to be able to determine whether TEQ levels in blood predict TEQ levels in liver. Patterns of individual PCB congeners in relation to biological factors and exposure are discussed elsewhere (Leonards et al., 1997a).

The response of the CALUX assay, using recombinant rat (H4IIE) hepatoma cell lines, was compared with the TEQ levels based on individual PCB concentrations determined using GC combined with electron capture detection (ECD) or ion trap detection (ITD). The correlation of the CALUX-TEQs with the so-called GC-TEQs was determined for otter liver and plasma samples, fish and sediment. The bioaccumulation of individual PCBs or PCBs expressed as TEQs via the route sediment → fish → otter, is described by Smit et al. (1996).

As plasma retinol and thyroid hormone cannot be measured in the hemolytic blood from dead otters, blood plasma was collected from captive otters. In this blood plasma retinol, total T4 (TT4), and free T4 (FT4) concentrations were determined. In addition, TEQ levels were determined through chemical analysis (using GC) and bio-analysis (with the CALUX assay) to obtain a measure of exposure.

2. Materials and methods

2.1. Chemicals

Diisopropylether, Tris, HCl, NaCl, *n*-hexane and 2,6-di-*tert*-butyl-4-methyl phenol (BHT) were of analytical grade and purchased from Merck (Germany). Methanol (HPLC quality) and ultra-clean dimethyl sulphoxide (DMSO) were purchased from Janssen Chimica (The Netherlands). The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard was purchased from Schmidt (The Netherlands) and the concentration of the stock was confirmed by GC–MS analysis (Dutch State Institute for Quality Control of Agricultural Products, RIKILT-DLO). Sulphuric acid (z.a., Becker) was washed three times with *n*-hexane before use in silica columns. Silica gel 60 (70–230 mesh ASTM, Merck) and Alumina B super I (ICN biomedicals, Eschwege, Germany) were activated before use by drying during 24 h at, respectively, 140 and 200°C.

2.2. Samples

2.2.1. Liver and blood from dead otters

Dead otters were collected from 1992 to 1993 in Denmark, and the health status was recorded (Leonards et al., 1997a). After these were analysed additional samples were selected to acquire a balanced distribution of PCB concentrations. A fast pre-screening of samples from Austria, Sweden and Scotland was performed with the CALUX assay, to enable selection of samples with varying TEQ-levels. Relatively fresh liver aliquots of 10–20 g from 12 otters were prepared separately for CALUX and chemical analysis (see below). Samples of about 1 g were prepared for hepatic retinoid analysis. Whenever possible, blood was collected from the carcasses for chemical and CALUX analysis.

2.2.2. Fish and sediment

In the five lakes in Denmark where most of the dead otters we used originated, 15 samples of fishes, representative for the otter diet, were collected in 1995. From each fish species approximately 25 individuals were homogenized using a blender and lyophilized before extraction (Smit et al., 1996). From each lake a representative sediment samples was collected, homogenized and dried at 60°C.

2.2.3. Blood plasma from living otters

Twenty-three blood plasma samples of 1–2 ml were obtained from a British otter rehabilitation institute in 1994 (ten samples) and 1995 (nine samples), and from an otter breeding centre in The Netherlands (four samples). In these plasma samples thyroid hormones, retinol and GC- and CALUX-TEQ levels were analysed.

2.3. Extraction and clean-up for CALUX and GC–MS analysis

Before extraction, tissue (liver, whole blood and fish) and sediment samples were mixed with anhydrous sodium sulphate. The sediment samples were freeze dried before extraction. All samples were extracted using a Soxhlet apparatus, during 6 h with 190 ml pentane/dichloromethane (1:1, v/v) for the tissues, and pentane/acetone (1:1, v/v) for the sediments at 50°C. The extract was evaporated to a volume of 5 ml in a Kuderna Danish setup at 70°C. In the tissue samples lipids were gravimetrically determined in 10% of the extract. The rest of the extract was cleaned over a multi-layer glass column (25 cm × 20 mm i.d.). For the tissue samples this column contained, from top to bottom: 0.5 cm anhydrous sodium sulphate and 10 g 33% H_2SO_4 deactivated silica on a bit of glass wool. For the sediment samples: 0.5 cm anhydrous sodium sulphate, 5 g 20% H_2SO_4 and 5 g 33% H_2SO_4 deactivated silica. The columns were vibrated for 30 s to pack them, rinsed with 15 ml hexane/diethyl ether (97:3, v/v), and eluted with 40 ml of the same elution fluid. The first 5 ml were discarded. The sediment extract was subsequently cleaned over a multi-layer column filled with alumina oxide (deactivated with 5% H_2O). The extracts were evaporated to almost 1 ml, under a gentle filtered air stream. Sulphur was removed from the sediment extracts using tetrabutylammonium sulphite as described by Murk et al. (1996a). Part of the extracts was used for the CALUX assay, the rest was fractionated over a silica gel (5% H_2O deactivated) column to remove pesticides.

For extraction of blood plasma, an equal amount of isopropanol was added, the mixture vortexed and left to stand for 10 min. Hexane was added in an amount twice that of the plasma and vortexed twice vigorously. The hexane and water layers were separated by centrifuging for 2 min at $3000 \times g$ in a bench top centrifuge. The upper (hexane) phase was collected in another glass tube using a pasteur pipette. One drop of concentrated HCl was added to the remaining water phase (one drop of HCl up to 500 μl original sample volume) and mixed vigorously. The hexane extraction was repeated twice as describe above, pooling the three hexane phases in another tube. After the third extraction step a fourth portion of about 200 μl hexane was

added to the remaining hexane on the water phase without disturbing the interphase and, without mixing, added to the hexane pool. The hexane was evaporated under a gentle filtered air stream, shortly before the sample was dry the desired amount of DMSO was added, and the rest of the hexane was evaporated again. The recovery of radio-labelled PCB-153 added to plasma 12 h before extraction using the method described above was $100 \pm 2\%$. If a sample was too small to handle easily, the plasma was diluted with demineralised water. The ideal sample size is 250–500 μl.

2.4. CALUX assay

Rat H4IIE.pGudluc1.1 cells, prepared as previously described (Aarts et al., 1995; Garrison et al., 1996), were grown in minimal essential medium (α-MEM, Gibco) with 10% heat-inactivated fetal calf serum (FCS, Gibco) and 500 μg of G418 (Geneticin, Gibco) per ml of medium, at a temperature of 37°C and 5% CO_2. The CALUX experiments with the liver extracts were performed in 24-well cell culture plates (Costar) and, due to technical improvements in the mean time, the other extracts were tested in 96-well cell culture plates (view plates, Packard), both in α-MEM without G418. For the CALUX assay performed in 24-well culture plates, cells were seeded in 500 μl growth medium and incubated for 24 h until the cell layer was 80–90% confluent (about 125000 cells/well). The medium was replaced by fresh growth medium containing the test compound in maximal 0.5% DMSO. After 24 h incubation the cells were rinsed twice with 50% diluted PBS ($0.5 \times$ PBS), 75 μl lysis mix was added, the cells were harvested 15 min later and centrifuged for 90 s. at $13000 \times g$. The supernatant was frozen at −80°C. For the CALUX assay performed in 96-well culture plates, the H4IIE.Luc cells were seeded in 100 μl growth medium, after 24 h incubation the cell layer was 80–90% confluent (about 20000 cells per well), and 100 μl of fresh medium containing the test compound in maximal 1% DMSO was added to each well. After an additional 24 h incubation, the plates were rinsed twice with $0.5 \times$ PBS. To each well 30 μl lysis buffer (10 mM Tris, 2 mM dithiolthreitol (DTT) and 2 mM 1,2,-diaminocyclohexane-*N*,*N*,*N'*,*N*-tetra-acetic acid, pH 7.8) was added, and after 10 min incubation on ice, the plates were placed at −80°C for at least 30 min.

For luciferase measurement either 20 μl supernatant was pipetted in a 96-well microtiter plate (24-well method) or the viewplate itself was used (96-well method). The microtiter plate was inserted in a Luminometer (Luminometer, Labsystems) and 100 μl of luciferin assay mix (Promega) was added. The light output was measured using flash kinetics.

Extracts were tested in triplicate, and to be able to correct for differences in quantification due to assay to assay variation, three TCDD calibration standards were measured with each assay. 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 in the area between the EC_5 and EC_{50}.

2.5. Chemical analysis

The cleaned extracts were further separated into three fractions containing non-, mono- and di-*ortho*-substituted PCBs, using a PYE HPLC column. The di- and mono-*ortho* fractions were measured with GC with electron-capture detection (ECD), the non-*ortho* fraction using GC with an ion-trap mass spectrometer detector (ITD), both according to Leonards et al. (1994b, 1997b). The here so-called GC-TEQs were calculated based on TEF values described by Ahlborg et al. (1994). PCB concentrations were also expressed as the total concentration of the seven standard congeners (Σ7PCB), which are PCBs 26, 52, 101, 118, 138, 153 and 180).

2.6. Analysis of retinoids

The analyses of hepatic and plasma retinoids were performed according to Brouwer et al. (1989c) with some modifications as described in Morse and Brouwer (1995). Briefly, for the extraction of hepatic retinoids 1–3-g pieces of liver were homogenized in 50 mM Tris–HCl buffer, pH 7.5. Aliquots of 50 μl liver homogenate or blood plasma were mixed with 50 μl methanol containing the internal standard (retinylacetate) and 0.1% BHT as an anti-oxidant. Plasma and liver samples were then extracted overnight with 100 μl diisopropylether at −20°C. The ether phase was collected and filtered over a 0.45-μm filter, then evaporated under nitrogen, and resuspended in 80 μl methanol with 0.1% BHT. Aliquots of 20 μl were analysed with a Merck–Hitachi HPLC using a reversed-phase silica C_{18} column (Pecosphere, 3 μm particle size, 3.3 cm length and 4.6 mm internal diameter). Retinoids were detected at a wavelength of 326 nm. Plasma extracts were analysed isocratically with 85% methanol and 15% water with a flow rate of 1 ml/min during 10 min. Hepatic retinoids were analysed using 85% methanol and 15% water for 1.5 min followed by a gradient to 100% methanol for 2.5 min, and subsequent elution of the retinyl esters at 100% methanol for 12 min. The column was re-equilibrated for 6 min after each sample, with 85% methanol and 15% water.

In the plasma extracts retinol was quantified, in the liver extracts retinol and retinyl palmitate.

2.7. Analysis of plasma thyroid hormones

Total thyroxine (TT4) and free thyroxine (FT4) concentrations were determined in, respectively, 10- and 25-μl aliquots of plasma, using a commercially available chemiluminescence immunoassay (Amerlite assay kits, Amersham, UK). Thyroid hormone concentrations were calculated from the luminescence data with the Securia program of Amersham.

3. Results

3.1. Retinoid and TEQ levels in dead otters

In the environmentally exposed dead otters the hepatic GC- and CALUX-TEQ levels based on non- and mono-*ortho* PCBs, ranged from 0.1 to 56 ng/g lipid. Hepatic retinylpalmitate concentrations, ranging from 20100 to 1 μg/g lipid (600–0.03 μg/g wet weight) were negatively correlated with these TEQ levels (Fig. 1a). Hepatic retinol concentrations ranged from 2611 to 4.1 μg/g lipid (94–0.1 μg/g wet weight). The negative correlations of hepatic retinol (Fig. 1b) and retinylpalmitate concentrations with TEQ levels were all comparable and statistically significant, either expressed on a lipid or on a fresh weight basis. Based on the fitted curve the no observed effect concentration (NOEC) and 90% effect concentration (EC_{90}) for retinol were 1 and 5 ng TEQ/g lipid, respectively, and for retinylpalmitate 2 and 5 ng TEQ/g lipid, respectively. The correlations with PCBs expressed as ng Σ7PCB showed comparable patterns but were less clear (data not shown). Although not very accurate, the EC_1 and EC_{90} levels expressed as TEQ values can be converted into the seven indicator PCBs (Σ7PCBs: CBs 28, 52, 101, 118, 138, 153 and 180) using field-calibrated regression values (Smit et al., submitted), resulting in an EC_1 value of 4 mg Σ7PCBs/kg lipid weight and an EC_{90} of 11 mg Σ7PCBs/kg lipid. The average lipid content of the liver was 4.2%.

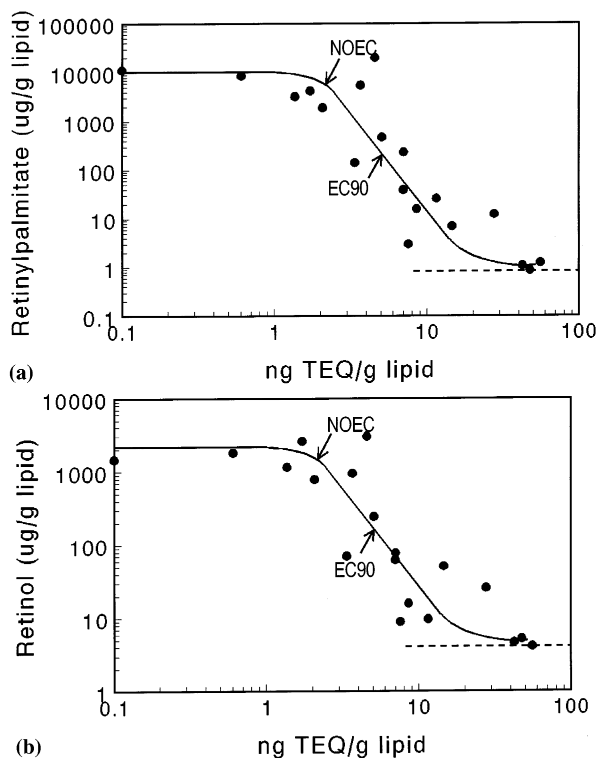

Fig. 1. (a) Hepatic retinylpalmitate levels (μg/g lipid) and (b) hepatic retinol levels (μg/g lipid) in environmentally exposed otters plotted against hepatic GC-TEQ levels (ng/g lipid; based on non- and mono-*ortho* PCBs). The calculated no observed effect concentration (NOEC) and 90% effect concentration (EC_{90}) for retinylpalmitate are 2 and 5 ng TEQ/g lipid, respectively (see arrows).

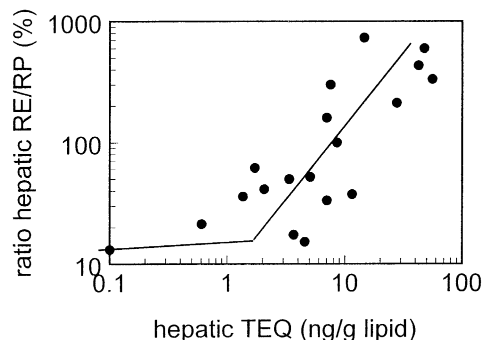

Fig. 2. The hepatic retinol/retinylpalmitate (RE/RP) ratio expressed as % (w/w) in environmentally exposed otters plotted against hepatic GC-TEQ levels (ng/g lipid; based on non- and mono-*ortho* PCBs).

Although both hepatic retinol and hepatic retinylpalmitate concentrations strongly decrease with increasing hepatic TEQ level, the ratio of hepatic retinol over retinylpalmitate increases from 13% in relatively clean, to 730% in highly exposed otters (Fig. 2). The strongest increase in ratio was found in otters with hepatic PCB concentrations of about 5 ng TEQ/g lipid and higher.

A statistically significant correlation was observed between hepatic CALUX-TEQ and hepatic GC-TEQ levels (Fig. 3). Hepatic GC-TEQ levels correlated with the GC-TEQ levels in blood from the same animals (Fig. 4). Unfortunately, insufficient data from the same animals were available to correlate CALUX-TEQs for liver and blood.

3.2. TEQs in fish and sediment samples

Correlations between CALUX-TEQ and GC-TEQ levels in fish samples also were statistically significant (Fig. 5a). The slope of this correlation, however, was 0.78 instead of 0.92 for otter liver samples. The TEQ levels were determined from only five sediment samples. Although the correlation was significant, the CALUX-TEQ was on average 26 times greater than the GC-TEQ (Fig. 5b).

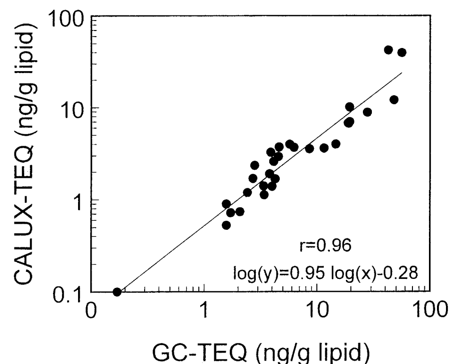

Fig. 3. Correlation between TEQ levels measured with the Ah receptor-mediated expression of the luciferase reporter gene (CALUX-TEQ) and calculated based on non- and mono-*ortho* PCB concentrations (GC-TEQs) in liver of environmentally exposed, dead otters ($P < 0.0001$).

3.3. Measurements in blood plasma of captive otters

In blood plasma of living, captive otters, the GC- and CALUX-TEQ levels range from 0.17 to 1.59 pg TEQ/ml plasma, with an average of 0.58 ± 0.36 pg TEQ/ml plasma. The CALUX-TEQs were determined in blood plasma samples of 100 μl, and more than half of the samples were found to be below the limit of detection (32 fg TEQ/well). As the rest of the blood plasma was used up already for the GC-TEQ determinations the CALUX-assay could not be repeated with somewhat larger amounts of plasma.

Plasma retinol concentrations ranged from 67 to 203 ng/ml plasma, with an average of 122 ± 38 ng/ml. When all animals were taken as one group, only a slight decrease in plasma retinol concentrations with increasing GC-TEQ levels was observed, which was not statistically significant (data not shown). Plasma TT4 (Fig. 6) and FT4 ($r = 0.48$, $P < 0.05$) increased significantly with increasing GC-TEQ levels. The blood plasma samples of the captive otters originated from three groups: a British group sampled in 1994, a British group sampled in 1995, and a group of otters sampled in The Netherlands. The average values of plasma GC-TEQ, retinol, TT4 and FT4 concentrations differ for these three groups (Table 1). Plasma retinol concentrations in these groups did not correlate with GC-TEQ levels.

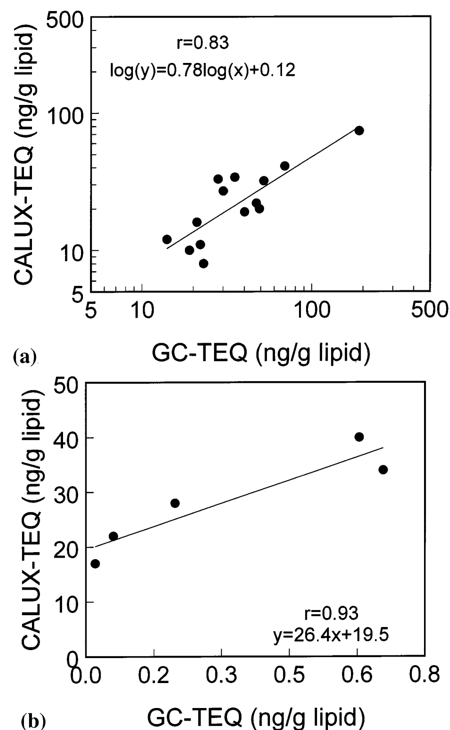

Fig. 5. Correlation between TEQ levels measured with the Ah receptor-mediated expression of the luciferase reporter gene (CALUX-TEQ) and calculated based on hepatic non-and mono-*ortho* PCB concentrations (GC-TEQs) in (a) extracts from fish homogenates ($P < 0.0001$) and (b) non-destructively cleaned sediment extracts ($P < 0.05$).

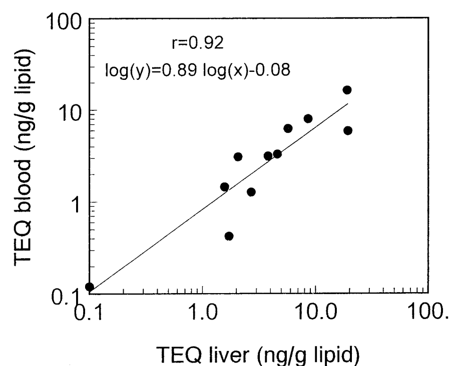

Fig. 4. Correlation between GC-TEQ levels (ng/g lipid; based on non- and mono-*ortho* PCBs) in liver and blood plasma of environmentally exposed otters ($P < 0.001$).

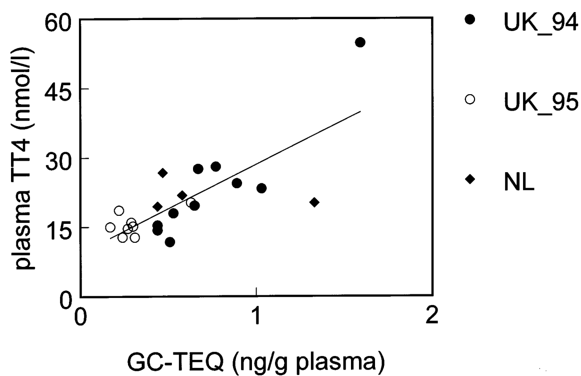

Fig. 6. Correlation between plasma TEQ levels (pg/ml plasma, based on non- and mono-*ortho* PCBs) and plasma total thyroxine (TT4) concentrations from captive otters. The origin of the samples is indicated with three different markers (UK, from a British rehabilitation centre in 1994 or 1995; NL, from a Dutch otter breeding centre).

Table 1
Average plasma GC-TEQ, retinol (RE), total thyroxine (TT4) and free thyroxine (FT4) concentrations (± standard deviation) in captive otters

	GC-TEQ (pg/ml)	Plasma RE (ng/ml)	Plasma TT4 (nmol/l)	Plasma FT4 (pmol/l)	*n*
Total group	0.58 ± 0.36	121 ± 38	20.4 ± 8.9	14.2 ± 4.4	23
UK 1994[a]	0.75 ± 0.35A[c]	124 ± 47	23.7 ± 12.2	16.2 ± 4.5A	10
UK 1995[b]	0.30 ± 0.13B	113 ± 19	16.1 ± 2.8	11.2 ± 3.1B	9
NL[b]	0.71 ± 0.42	136 ± 53	22.0 ± 3.3	15.2 ± 3.8	4

n is the number of otters in each group.
[a] UK, otters from a British rehabilitation centre.
[b] NL, otters from a Dutch otter breeding centre.
[c] Group averages are differ statistically significant ($P < 0.05$) from all group averages indicated with a different letter.

4. Discussion

4.1. AhR-related decrease in retinoid concentrations

These results suggest that otters are sensitive to compounds acting via the AhR. In combination with the selective accumulation by otters of the most toxic, planar PHAHs (Leonards et al., 1997b), current environmental concentrations appear to be high enough to almost cause an retinoid depletion. Although dietary intake of vitamin A can also influence hepatic vitamin A concentrations, disturbances in vitamin A homeostasis have also been associated with PHAH exposure in experimental setups where the food quality was kept constant for all experimental groups, for example doves (Spear et al., 1989), harbour seals (Brouwer et al., 1989c), eider ducks (Murk et al., 1994) and mink (Brunström et al., 1991). The increased ratio of retinol over retinylpalmitate with internal exposure observed in the feral otters, suggests that either the storage capacity or the mobilisation of retinoids was disturbed in a dose-related manner. In the study described by Brunström et al. (1991) the hepatic vitamin A content was reduced by 48% in adult female mink fed 2 mg Clophen A50 during 12–14 weeks. The vitamin A concentration was determined after complete hydrolyses, so it was not possible to distinguish between retinyl esters and retinol. An important difference with feral otters was that the mink were fed vitamin supplements, which may at least partially compensate the adverse effects of the PCBs on vitamin A homeostasis. Nevertheless, in the lungs of the same animals vitamin A concentrations were reduced by 67%. This shows that in a target tissue vitamin A concentrations can already be reduced before the liver, the main vitamin A storage organ, is depleted. In Sprague–Dawley rats dosed once with 10 ng 2,3,7,8-TCDD/g body weight, retinoids were mobilized from hepatic and extrahepatic storage sites already within 14 days after exposure. Retinylpalmitate concentrations were decreased in liver (to 2.4%) and lung (to 20%), whereas retinyl palmitate and retinol concentrations were increased in kidney (to 850 and 245%, respectively) and retinol concentrations in serum (to 145%) (Brouwer et al., 1989c).

4.2. Possible consequences

Leonards et al. (1997a) studied the frequency and severity of diseases in feral Danish otters, and found that these increased with increasing hepatic TEQ levels (either determined with GC or CALUX assay). They divided the animals into three exposure groups of comparable size: low (0–5 ng TEQ/g lipid), middle (5–10 ng TEQ/g lipid) and high (> 10 ng TEQ/g lipid). Table 2 presents the average hepatic TEQ levels and retinoid concentrations for all animals tested in this study, divided into the same exposure groups as Leonards et al. (1997a). The concentration ranges at which increased disease rates occurred, correlated with ranges at which the hepatic retinol and retinylpalmitate concentrations decreased. This was to be expected, as vitamin A is not only essential for normal growth and development, but also for resistance against infections. There is a bidirectional relationship between vitamin A deficiency and increased infection, which may result in a vicious cycle: vitamin A deficiency increases the risk for infection, which in turn decreases vitamin A concentrations (Sommer et al., 1984). Vitamin A functions in maintaining anatomical barriers of the body against microbial colonization and infection, especially the epithelial lining of the respiratory, genitourinary and gastrointestinal tracts. Vitamin A also influences the systemic immune response, including antibody production and T lymphocyte proliferation and activity (Davis and Sell, 1989; Friedman and Sklan, 1989; Sijtsma et al., 1989). Immune responses were often affected before other manifestations of vitamin A deficiency were observed.

In a Danish survey for the health status of feral otters, especially pneumonia was a frequently occurring infectious disease, while this was never registered for otters living in captivity (Madsen, 1996). This difference could be caused by a combination of a lower PCB exposure, less challenging conditions of living, and addition of vitamin A supplements to the food. As a consequence, animals living in captivity will not so easily be a victim of the viscous cycle mentioned above, and therefore experimental studies with wildlife species may result in an underestimation of the risks involved.

Table 2
Average GC-TEQ, hepatic retinol and retinylpalmitate concentrations, the average ratio hepatic retinol (RE) over retinylpalmitate (RP) (all ± standard deviation), and infection incidence in environmentally exposed dead otters grouped after their exposure levels

GC-TEQ ranges (ng/g lipid)	GC-TEQ (ng/g lipid)	RE (μg/g liver)	RP (μg/g liver)	Ratio RE/RP (%)	Infection incidence[a]	*n*
0–5	2.2 ± 1.6A[b]	58 ± 34A	273 ± 238A	32 ± 18A	Low	8
5–10	7.0 ± 1.3A	2.4 ± 2.9B	4.4 ± 5.9B	129 ± 107B	Middle	5
>10	33.2 ± 18.1B	0.4 ± 0.4C	0.2 ± 0.3C	392 ± 254B	High	6

n is the number of otters in each group.
[a] More information on the infection incidence is described in Leonards et al. (1997a).
[b] Statistically significant ($P<0.05$) different group averages are indicated with different letters.

4.3. CALUX-TEQ levels as a measure of internal dose

The almost linear, strong correlation between hepatic CALUX-TEQ and hepatic GC-TEQ levels indicates the TEF values chosen for calculation of the GC-TEQ levels are a good measure of the toxic potency of these PCBs, which was measured directly in the CALUX assay. Former experiments, with single compounds, already indicated a good correlation between the TEF values of several PCDDs, PCDFs and PCBs as proposed by Ahlborg et al. (1994) and the toxic potency relative to TCDD as measured in the CALUX assay (Aarts et al., 1995; Garrison et al., 1996; Sanderson et al., 1996).

The good correlation between blood and hepatic TEQ levels indicates that TEQ levels in blood samples can be used as a measure for internal dose, in this case TEQ levels in livers. This is in accordance with earlier results with experimentally exposed eider ducks, describing a good correlation between CALUX-TEQ levels in blood plasma and PCB levels in abdominal lipids (Murk et al., 1997). The possibility to determine the internal dose based on a blood sample offers the possibility of non-destructive monitoring of exposure.

4.4. CALUX-TEQs in fish and sediment samples

Fish extracts contain a quite different patten of PCBs than otters, with relatively much di-*ortho* PCBs (Leonards et al., 1997b). This means that quite different PCBs contributed to the GC-TEQs in the fish samples compared to the otters. The TEFs of PCBs are mainly based on cytochrome P450 IA induction relative to TCDD, measured as the EC_{50} for ethoxyresorufin deethylase (EROD) activity in H4IIE hepatoma cells. It is, however, difficult to determine the EC_{50} in an EROD assay of less potent PCBs because of the phenomenon of substrate inhibition (Murk et al., 1996a,b). This could explain the difference between the calculated GC-TEQ and the measured CALUX-TEQ. A solution could be using TEF values determined in the CALUX assay, as this assay is not hindered by substrate inhibition (Murk et al., 1996a,b). It cannot yet be excluded that mixture interactions contribute to the discrepancy between the estimated and the measured toxic potency of the mixture. Species-specific antagonism of some di-*ortho* PCBs on the induction by planar PHAHs has been demonstrated to occur at the level of the AhR (Aarts et al., 1995; Richter et al., 1997) and the fish extracts contain relatively high concentrations of di-*ortho* PCBs. For a worst-case indication of the TEQs in a mixture, CALUX cell lines of species without antagonism could be used, e.g. guinea pig (GPC16) or man (HEPG2) (Aarts et al., 1995). Another option is to develop a CALUX cell line for a specific species of interest, in this case the otter, to be able to directly measure the toxicological risk of the mixture of PHAHs in the food of this species.

The sediment extracts used have been cleaned using an aluminum column, a common procedure for chemical analysis, and not with a silica column containing sulphuric acid as is standard in the clean-up procedure for the CALUX assay (Murk et al., 1996a). An aluminium column is not destructive for poly-aromatic hydrocarbons (PAHs), so these will still be present in the extract tested in the CALUX assay. Several PAHs can bind to and activate the AhR, and induce, for example, cytochrome P450 IA. These PAHs will also induce CALUX activity. As the calculations of the GC-TEQs were only based on PCB concentrations, they were expected to be lower than the CALUX-TEQ. In addition to PAHs, other unidentified AhR-active compounds probably were present in the sediment extracts as well. Although a linear fit was used in Fig. 5b, a saturation curve was expected to be more appropriate. To become sure about this, more, especially clean, sediment samples would have to be tested. For quantification of the PHAHs only, a destructive clean-up with separation of the PHAHs from the PAHs is currently in use (Murk et al., in preparation).

4.5. Measurements in blood plasma of captive otters

The GC- and CALUX-TEQ levels of the captive otters were low compared to the levels found in liver and whole blood of the dead otters. Assuming an average blood lipid content of 0.25%, these ranges would be 0.07–0.64 ng TEQ/g lipid, with an average of

0.23 ± 0.14 ng TEQ/g lipid. The CALUX-TEQs were determined in blood plasma samples of 100 μl, and more than half of the samples were found to be below the limit of detection. This detection limit was 0.1 fmol TEQ/well or 32 fg TEQ/well. With the use of 100 μl of plasma and the samples tested in triplicate, the extract of 25 μl blood plasma was tested in each well, so the detection limit was 1.28 pg/ml plasma. With the use of 500 μl plasma this limit would have been 0.2 pg TEQ/ml, low enough to be able to quantify most of these relatively clean samples.

The relatively low TEQ levels in the captive otters probably were not high enough to significantly reduce plasma retinol concentrations. As plasma retinol concentrations can be influenced by PCBs in two opposite manners, initially increased as a result of reduced storage capacity in the liver and decreased as a result of binding of hydroxylated metabolites (OH-PCBs) to TTR, plasma retinol concentrations will only be significantly reduced after relatively high PCB exposure. In addition, homeostasis will strictly regulate plasma retinol concentration as long as possible. Of course, vitamin supplements would also mask vitamin effects.

Although a significant correlation was observed between plasma GC-TEQ and thyroid hormone concentrations in the captive otters (Fig. 6), it can at present not be decided whether these correlations were a result of a causal relationship, of a co-correlation with a possibly not PCB-related factor such as food quality or dwelling place, or a coincidence because of the different ambient temperatures or moments of sampling. The correlation between TT4 or FT4 and GC-TEQ levels for only the two British groups was stronger ($r = -0.89$, $P < 0.0001$ and $r = 0.62$, $P < 0.01$, respectively). PCBs have been reported to influence plasma thyroid hormone concentrations directly via OH-PCBs, or indirectly via effects on the thyroid hormone metabolism. Increased plasma concentrations of OH-PCBs will decrease levels of thyroid hormone bound to TTR (Lans et al., 1994), resulting in increased amounts of unbound, free T4. The ratio free over total T4, however, did not increase with GC-TEQ levels (data not shown). PCBs have also been reported to alter thyroid hormone metabolism, for example via reduction of deiodinase activity, resulting in lower concentrations of the active form of thyroid hormone, triiodothyronine (T3), or via induction of thyroid hormone glucuronidation which would result in enhance hepatic elimination of T4 or T3 (Beetstra et al., 1991; Barter and Klaassen, 1992; Visser et al., 1993). The thyroid gland, however, will synthesize more T4 upon thyroid hormone reduction, in an attempt to maintain thyroid hormone homeostasis in the blood. Slight disturbances of thyroid hormone homeostasis could only be observed by measuring enzyme induction or thyroid-stimulating hormone (TSH) concentrations. TSH concentrations will also fluctuate depending on requirements of the animal to adapt to change in, for example, external temperature or season (Hulbert, 1985; Eales and Shostak, 1986; Brigmaon et al., 1992). Enzyme activities cannot be studied in a non-destructive manner, as this would require fresh liver samples. In an experimental study with mink exposed via their diet to carp from Saginaw Bay, Michigan, both total and free plasma T4 concentrations increased with increasing TEQ-levels in their food, while total and free T3 decreased (Heaton et al., 1995). Although these changes were not statistically significant, they suggest a reduction in T4-deiodinase activity. Unfortunately the deiodinase activities were not reported to be measured.

5. Conclusions

(1) A strong negative correlation was observed between hepatic vitamin A concentrations and TEQ levels in environmentally exposed European otters. These results indicate that otters are sensitive for AhR-related toxic effects of PCBs, and that current environmental PCB concentrations are high enough to cause adverse effects.

(2) The TEQ levels in the otter blood can be used to predict the TEQ levels in otter liver.

(3) Otters with an internal dose of more than 2 ng TEQ/g lipid, either measured in liver or in blood plasma, had strongly reduced hepatic retinoid concentrations, which coincided with a higher incidence of infectious diseases. The EC_{90} level for reduced hepatic retinoid concentrations was 5 ng TEQ/g lipid.

(4) Although not very accurate, the TEQ values are converted into the seven indicator PCBs (Σ7 PCBs: CBs 28, 52, 101, 118, 138, 153 and 180) using field-calibrated regression values (Table 3). To assist in risk estimations, the TEQ levels in the diet of the otter and sediment collected on the same locations as the otters is added. The exact information about the calculated otter–fish biomagnification factors and fish–sediment accumulation factors determined can be found elsewhere (Smit et al., submitted).

(5) The toxicological potency of PCBs acting via the AhR, expressed as CALUX-TEQs, correlated well with the GC-TEQ levels estimated based on chemical PCB measurements both in otter liver and fish samples.

(6) The CALUX-TEQ of non-destructively cleaned sediments extracts was 26 times greater than the GC-TEQ based on PCB concentrations. This difference can be explained by the presence of PAHs in the extracts.

(7) Internal PCB exposure, expressed as GC- or CALUX-TEQs, of otters living in captivity, was lower than the internal dose of the dead otters that were used for this study.

Table 3
EC_1 and EC_{90} levels for hepatic vitamin A reduction in otter liver expressed as TEQs and Σ7PCBs

	Hepatic vitamin A reduction	TEQs in otter liver[a] or blood plasma (ng/g lipid)	Σ7PCBs[b] in otter liver or blood plasma (mg/kg)	TEQs in fish (total diet[c]) (ng/kg lipid)	TEQs in sediment (ng/kg org-C)
Safe level	EC_1	2	4	11	3
Critical level	EC_{90}	5	11	29	7

Also presented are the TEQ-levels in the diet of the otter and sediment collected on the same locations where the otters came from. More information about the calculated otter–fish biomagnification factors and fish–sediment accumulation factors determined will be presented elsewhere (Smit et al., submitted).
[a] Average lipid content 4.2%.
[b] TEQ values converted into the seven indicator PCBs (Σ7PCBs: CBs 28, 52, 101, 118, 138, 153 and 180).
[c] Average lipid content 6.2%
[d] In Dutch standard sediment, organic carbon averages 5.72% of dry weight.

(8) Plasma retinol concentrations of relatively clean captive otters did not correlate with plasma GC-TEQ levels.

(9) Although plasma total and free thyroid hormone concentrations in the relatively clean captive otters were positively correlated with plasma GC-TEQ levels, it cannot yet be concluded that these correlations were due to a causal relationship.

(10) The internal dose expressed as TEQs of relatively clean otters can be quantified with the CALUX assay using 0.5 ml of blood plasma. For determination of whether an otter has an internal dose of less than 2 ng TEQ/g lipid (5 pg TEQ/ml plasma), and for quantification of higher TEQ levels, an aliquot of 50 μl blood plasma was more than enough.

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References

Aarts, J.M.M.J.G., Denison, M.S., Cox, M.A., Schalk, M.A.C., Garrison, P.A., Tullis, K., De Haan, L.H.J., Brouwer, A., 1995. Species-specific antagonism of Ah receptor action by 2,2′,5,5′-tetrachloro- and 2,2′,3,3′,4,4′-hexachlorobiphenyl. Eur. J. Pharm. Env. Tox. Sect. 293, 463–474.

Ahlborg, U.G., Becking, G.C., Birnbaum, L.S., Brouwer, A., Derks, H.J.G.M., Feeley, M., Golor, G., Hanberg, A., Larsen, J.C., Liem, A.K.D., Safe, S.H., Schlatter, C., Waern, F., Younes, Yränheikki, E., 1994. Toxic equivalency factors for dioxin-like PCBs. Chemosphere 28, 1049–1067.

Barter, R.A., Klaassen, C.D., 1992. UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extrathyroidal mechanism. Toxicol. Appl. Pharmacol. 113, 36–42.

Beetstra, J.B., Van Engelen, J.G.M., Karels, P., Van der Hoek, H.J., De Jong, M., Docter, R., Krenning, E.P., Hennemann, G., Brouwer, A., Visser, T.J., 1991. Thyroxine and 3,3′,5-triiodothyronine are glucuronidated in rat liver by different uridine diphosphate-glucuronyltransferases. Endocrinology 128, 741–746.

Brigmaon, R.L., Besch, E.L., Mather, F.B., 1992. Seasonal temperature and its influence on plasma corticosterone, triiodothyronine, thyroxine, plasma protein and packed cell volume in mature male chickens. Comp. Biochem. Physiol. 102a, 289–293.

Broekhuizen, S., 1989. Belasting van otters met zware metalen en PCBs. Levende Natuur (in Dutch) 90 (2), 43–47.

Brouwer, A., 1991. The role of enzymes in regulating the toxicity of xenobiotics. Role of biotransformation in PCB-induced alterations in vitamin A and thyroid hormone metabolism in laboratory and wildlife species. Biochem. Soc. Trans. 19, 731–738.

Brouwer, A., Van den Berg, K.J., 1986. Binding of a metabolite of 3,4,3′,4′-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxin. Toxicol. Appl. Pharmacol. 85, 301–312.

Brouwer, A., Hakansson, H., Kukler, A., van den Berg, K.J., Ahlborg, U.G., 1989a. Marked alterations in retinoid homeostasis of Sprague–Dawley rats induced by a single i.p. dose of 10 μg/kg of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicology 58, 267–283.

Brouwer, A., Reijnders, P.J.H., Koeman, J.H., 1989b. Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). Aquatic Toxicol. 15, 99–106.

Brouwer, A., Hakansson, H., Kukler, A., van den Berg, K.J., Ahlborg, U.G., 1989c. Marked alterations in retinoid homeostasis of Sprague–Dawley rats induced by a single i.p. dose of 10 μg/kg of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicology 58, 267–283.

Brouwer, A., Lans, M.C., De Haan, L.H.J., Murk, A.J., Morse, D.C., 1994. Formation and toxicological aspects of phenolic metabolites of polychlorobiphenyls (PCBs) and related compounds. Organohalogen Compounds 20, 465–469.

Brunström, B., Hakansson, H., Lundberg, K., 1991. Effects of a technical PCB preparation and fractions thereof on ethoxyre-

sorufin *O*-deethylase activity, vitamin A levels and thymic development in the Mink (*Mustela vison*). Pharmacol. Toxicol. 69, 421–426.

Chen, L.-C., Berberian, I., Koch, B., Mercier, M., Azais-Braesco, V., Glauert, H.P., Chow, C.K., Robertson, L.W., 1992. Polychlorinated and polybrominated biphenyl congeners and retinoid levels in rat tissues: structure-activity relationships. Toxicol. Appl. Pharmacol. 114, 47–55.

Davis, C.Y., Sell, J.L., 1989. Immunoglobulin concentrations in serum and tissues of vitamin A-deficient broiler chicks after newcastle disease virus vaccination. Poultry Sci. 68, 16–144.

Denison, M.S., El-Fouly, M.H., Arts, J.M.M.J.G., Brower, A., Richter, C., Giesy, J.P., 1993. Production of novel recombinant cell line bioassay systems for detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like chemical, Short paper Dioxin 1993. Organolhalogen Compounds, 365–368. et al., 1993.

DeVito, M.J., Birnbaum, L.S., 1994. Toxicology of dioxins and related chemical. In: Schecter, A. (Ed.), Dioxins and Health. Plenum Press, New York, pp. 139–162.

Eales, J.G., Shostak, S., 1986. Influences of temperature and pH on free T4 and free T3 in charr and trout plasma. Gen. Comp. Endocrinol. 61, 272–277.

Friedman, A., Sklan, D., 1989. Antigen-specific immune response impairment in chick as influenced by dietary vitamin A. J. Nutr. 119, 790–795.

Heaton, S.N., Bursian, S.J., Giesy, J.P., Tillitt, D.E., Render, J.A., Jones, P.D., Verbrugge, D.A., Kubiak, T.J., Aulerich, R.J., 1995. Dietary exposure of mink to carp from Saginaw Bay, Michigan; 1. Effects on reproduction and survival, and the potential risks to wild mink populations. Arch. Environ. Contam. Toxicol. 28, 334–343.

Garrison, P.A., Tullis, K., Aarts, J.M.M.J.G., Brouwer, A., Giesy, J.P., Denison, M.S., 1996. Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxine-like chemicals. Fund. Appl. Toxicol. 30, 194–203.

Goldstein, J.A., Safe, S., 1989. Mechanism of action and structure-activity relationships for the chlorinated dibenzo-*p*-dioxins and related compounds. In: Kimbrough, Jensen (Eds.), Halogenated Biphenyls, Terphenyls, Naphtalenes, Dibenzodioxins and Related Products. Elsevier, Amsterdam. pp. 239–294.

Hulbert, A.J., 1985. A comparative study of thyroid function in reptiles and mammals. In: Follett, B.K., Chandola, A. (Eds.), The Endocrine System and the Environment. Jap. Sci. Press, Tokyo/ Springer-Verlag, Berlin, pp. 105–115.

Jensen, R.K., Cullum, M.E., Deyo, J., Zile, M.H., 1987. Vitamin A metabolims in rats chronically treated with 3,3′,4,4′,5,5′-hexabromobiphenyl. Biochim. Biophys. Acta 926, 310–320.

Jensen, S., Kihlström, J.E., Olsson, M., Lundberg, C., Örberg, J., 1977. Effects of PCB and DDT on mink (*Mustela vison*) during the reproductive season. Ambio 6, 239.

Lans, M.C., Klason-Wehler, E., Willemsen, M., Meussen, E., Safe, S., Brouwer, A., 1993. Stucture dependent competitive interaction of hydroxychlorobiphenyls, dibenzo-*p*-dioxins and dibenzofurans with human transthyretin. Chem. Biol. Interact. 88, 7–21.

Lans, M.C., Spiertz, C., Brouwer, A., Koeman, J.H., 1994. Different competition of thyroxine binding to transthyretin and thyroxine binding globulin by hydroxy-polychlorobiphenyls, -dibenzo-*p*-dioxins and -dibenzofurans. Eur. J. Pharmacol., Environ. Toxicol. Pharmacol. Sect. 270, 129–136.

Leonards, P.E.G., Smit, M.D., De Jongh, A.W.J.J., Van Hattum, B., 1994a. Evaluation of Dose-response Relationships for the Effects of PCBs on the Reproduction of Mink (*Mustela vision*). VU Boekhandel/Uitgeverij, Amsterdam, 47 pp.

Leonards, P.E.G., Van Hattum, B., Cofino, W.P., Brinkman, U.A.Th., 1994b. The occurrence of non-ortho, mono-ortho and di-ortho substituted PCB congeners in different organs and tissues of polecats (*Mustela putorius* L.) from The Netherlands. Environ. Toxicol. Chem. 13, 129–142.

Leonards, P.E.G., Elmeros, M., Cofino, W.P., Conroy, J., Gutleb, A., Mason, C., Murk, A.J., Olsson, M., Smit, M., Van Hattum, B., Madse, A.B., 1997a. Toxic PCBs in European otter populations in relation to biological factors and health status. Environ. Pollut. (submitted).

Leonards, P.E.G., Zierikzee, Y., Brinkman, U.A.Th., Cofino, W.P., Van Straalen, N.M., Van Hattum, B., 1997b. The selective dietary accumulation of planar polychlorinated biphenyls in the otter (*Lutra lutra*). Environ. Toxicol. Chem. 9 (16), 1807–1815.

Madsen, A.B., Odderens Luta lutra Okologi og forvaltning i Danmark. The ecology and conservation of the otter Lutra lutra in Denmark. PhD thesis Madsen. National Enviromental Research Institute, Department of Landscape Ecology, Ronde, Denmark. pp. 84. ISBN 87-7772-275-2.

Mason, C.F., 1989. Water pollution and otter distribution: a review. Lutra 32 (2), 97–131.

McConnell, E.E., 1985. comparative toxicity of PCBs and related compounds in various species of animals. Environ. Health Perspect. 60, 29–33.

McConnell, E.E., 1989. Acute and chronic toxicity and carcinogenesis in animals. In: Kimbrough, Jensen (Eds.), Halogenated Biphenyls, Terphenyls, Naphtalenes, Dibenzodioxins and Related Products. Elsevier, Amsterdam. pp. 161–193.

Morse, D.C., Brower, A., 1995. Fetal; neonatal and longterm alterations in hepatic retinoid levels following maternal polychlorinated biphenyl exposure in rats. Toxicol. Appl. Pharmacol. 131, 175–182.

Murk, A.J., Van den Berg, J.H.J., Fellinger, M., Rozemeijer, M.J.C., Swennen, C., Duiven, P., Boon, J.P., Brouwer, A., Koeman, J.H., 1994. Toxic and biochemical effects of 3,3′,4,4′-tetrachlorobiphenyl (CB-77) and Clophen A50 on eider ducklings (*Somateria mollissima*) in a semi-field experiment. Environ. Pollut. 86, 21–30.

Murk, A.J., Legler, L., Denison, M.S., Giesy, J.P., Van der Guchte, C., Brouwer, A., 1996a. Chemical Activated Luciferase Expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. Fund. Appl. Toxicol. 33 (1), 149–160.

Murk, A.J., Leonards, P.E.G., Jonas, A., Denison, M.S., Brouwer, A., 1996b. Application of the CALUX (chemical activated luciferase gene expression) assay for measuring TCDD-equivalents in sediment, pore water and blood plasma samples. Organohalogen Compounds 29, 64–69.

Murk, A.J., Leonards, P.E.G., Bulder, A.S., Jonas, A., Rozemeijer, M.J.C., Denison, M.S., Koeman, J.H., Brouwer, A., 1997. The CALUX (chemical-activated luciferase expression) assay adapted and validated for measuring TCDD equivalents in blood plasma. Environ. Toxicol. Chem. 16 (8), 1583–1589.

Nord, 1992. Risk assessment of polychlorinated biphenyls (PCBs). Nord 26, 1–99.

Olsson, M.L., Sandegren, F., 1983. Is PCB partly responsible for the decline of the otter in Europe? Proc. 3rd International Ottersymposium, Strasbourg, 24–27 November 1983. In: Reuther, C., Röchert, R. (Eds.), Proc. V Int. Otter Colloqium, Habitat 6, Hankesbüttel, 1991. pp. 223–227.

Richter, C.A., Giesy, J.P., Tieber, V.L., Denison, M.S., 1997. An in vitro rainbow trout cell bioassay for AhR-mediated toxins. Environ. Toxicol. Chem. 16, 543–550.

Safe, S., 1987. Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs), support for the use of the in vitro AHH induction assay. Chemosphere 16, 791–802.

Safe, S., 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzo furans (PCDFs), and related compounds: Environmental and mechanistic considerations wich support the development of toxic equivalence factors (TEFs). Crit. Rev. Toxicol. 21 (10), 51–88.

Sanderson, J.T., Aarts, J.M.M.J.G., Brouwer, A., Froese, K.L., Denison, M.S., Giesy, J.P., 1996. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin *O*-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicol. Appl. Pharmacol. 137, 316–325.

Sijtsma, S.R., West, C.E., Rombout, J.H.W.M., van der Zijpp, A.J., 1989. Vitamine A en Newcastle disease virusinfectie bij kippen. Voeding 50, 182–187. (Dutch).

Sommer, A., Katz, J., Tarwotio, I., 1984. Increased risk of respiratory disease and diarrea in children with preexisting mild vitamin A deficiency. Am. J. Clin. Nutr. 40, 1090–1095.

Smit, M.D., Leonards, P.E.G., Madsen, A.B., Van Hattum, B.G.M., Murk, A.J., De Jongh, W.J.J., 1996. Bioaccumulation of PCBs in Danish otterhabitats. DOQOP-report, ch. 2.

Spear, P.A., Garcin, H., Narbonne, J.F., 1988. Increased retinoic acid metabolism following 3,3′,4,4′,5,5′-hexabromobiphenyl injection. Can. J. Physiol. Pharmacol. 66, 1181–1186.

Spear, P.A., Bourbonnais, D.H., Peakall, D.B., Moon, T.W., 1989. Dove reproduction and retinoid (vitamin A) dynamics in adult females and their eggs following exposure to 3,3′,4,4′-tetrachlorobiphenyl. Can. J. Zool. 67, 908–913.

Van der Weiden, E.J., Hendriks, A.J., Klein, M.H.J., Leonards, P.E.G., Smit, M., Murk, A.J., Van de Plassche, E., Crommentuijn, T., Luttik, R., Van Hattum, B., 1996. Development of Otter-based quality objectives for PCBs (DOQOP): Overview of the project in view of the environmental policy aims. Organohalogen Compounds 29, 126–131.

Visser, T.J., Kaptein, E., Van Raaij, J.A.G.M., Tjong Tjin Joe, C., Ebner, T., Burchell, B., 1993. Multiple UDP-glucuronyltransferasesfor the glucuronidation of thyroid hormone with preference for 3,3′,5′-triiodotyronine (reverse T3). FEBS Lett. 315, 65–68.

Zile, M.H., 1992. Vitamin A homeostasis endangered by environmental pollutants. Proc. Soc. Exp. Biol. Med. 201, 141–153.