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**Regulation of Cytochrome P450 enzymes in H4IIE: A tool for
detection of dioxin-like activity and metabolic activation for
screening of estrogenicity**

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List of abbreviations

μg	<i>microgram</i>
μL	<i>microliter</i>
2,3,7,8, TCDD	<i>2,3,7,8, Tetrachlorodibenzo-p-dioxin</i>
AHH	<i>Aryl hydrocarbon hydroxylase</i>
AhR	<i>Ah-Receptor</i>
BPA	<i>Bisphenol A</i>
CPRG	<i>Chlorophenol-β-Galactosidase</i>
C-WHO	<i>Chemical-WHO</i>
CYP	<i>Cytochrome P450-Isoform</i>
DEE	<i>Diethyl ether</i>
DMEM	<i>Dulbecco's Modified Eagle Medium</i>
DMSO	<i>Dimethylsulfoxid</i>
DRE	<i>Dioxin Responsive Elements</i>
EDC	<i>Endocrine Disputers Compounds</i>
EPA	<i>Environmental Protection Agency</i>
ER	<i>Estrogen Receptor</i>
EROD	<i>Ethoxy-O-Resorufin-Deethylase</i>
FCS	<i>Fetal Calf Serum</i>
HAH	<i>Halogenated Aromatic Hydrocarbons</i>
hsp 90	<i>Heat shock proteins</i>
HRGC/HRMS	<i>High Resolution Gas Chloromatography/High Resolution Mass Spectroscopy</i>
TEF	<i>Toxicity Equivalent Factor</i>
I-TEQ	<i>International Toxicity Equivalent</i>
kg	<i>kilogram</i>
min	<i>minute</i>
mL	<i>milliliter</i>
n.d.	<i>not detected</i>
ng	<i>nanogram</i>
pg	<i>picogram</i>
nM	<i>nano Mole</i>
nm	<i>Nanometer</i>
PAH	<i>Polyaromatic Hydrocarbon</i>
PBS	<i>Phosphate Buffered Saline</i>
PCB	<i>Polychlorinated biphenyls</i>
PCDD/F	<i>Polychlorinated dibenzo-p- dioxin/furan</i>
pM	<i>Pico Mol</i>
POP	<i>Persistent Organic Pollutant</i>
Rba	<i>biological value/analytical value</i>
S1	<i>Genetic Engineering</i>
SOP	<i>Standard Operating Protocol</i>
TBA	<i>2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole and 2,4,6-tribromoanisole</i>
v/v	<i>volume per volume</i>
WHO	<i>World Health Organization</i>

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SUMMARY

The aim of the present thesis was the investigation of Cytochrome P450 regulation in two aspects.

The Enzyme induction was used as end point for dioxin-like compounds screening in different relevant matrices, using micro-EROD bioassay. In order to validate the use of this bioassay to determine the levels of dioxin-like compounds in food, we have participated in the first and second round of "Interlaboratory comparison of dioxin-like compounds in food using bioassays." Following matrices have been analyzed: biological samples (salmon, cod liver), environmental samples (Fly ash and fly ash extracts), PCDD/F and PCB mixture. The results were compared with those obtained in other laboratory assays and analytical data. The micro-EROD assay results showed good correlation with the chemical analysis; the exception was in the case of fly ash, due to the extraction procedure.

Furthermore, the performance of our assay for screening of different relevant matrices was investigated. In the case of leachate-polluted groundwater sample, the EROD assay indicates the ability of compounds to act as dioxin-like agonists. The samples were tested as crude extract and in parallel employing a sample preparation (where only the persistent dioxin-like compounds were detected). This assay showed a weak signal in the crude fraction which corresponds to a concentration of TEQ equivalents of 96pg and 23pg TEQ/L in the fraction which contains the persistent dioxin-like compounds only.

The estrogen assay was employed to test the raw water and extracts of the water prepared with n-hexane, dichloromethane, and benzene to cover different polarities of compounds. In this test, the extracts of n-hexane and benzene were negative, whereas the raw water and the dichloromethane extract resulted in positive weak signals, which correspond to a water concentration in terms of 17- β -estradiol equivalents of 4.8 nM and 3 pM respectively. After extraction, the active compounds were more present in the dichloromethane fraction.

Additionally, the activity of a natural halogenated organic compounds in EROD bioassay was evaluated, in agreement with the physico-chemical parameters determination of Q1 and TBA, the EROD induction potency of these compounds,

which typically (though not always) is a function of planarity of TCDD-like compounds was low. In conclusion, Q1 is not a planar potent molecule.

Finally, the use of EROD Bioassay as a bio-monitoring tool of dioxin-like compounds in mother milk was investigated and compared with analytical data. Two sample series from Denmark and Finland were tested; generally, it could be observed that the EROD-TEQ values correlate well but still lower than those from chemical analysis. This difference is higher for the Finnish samples than for Denmark, the results suggest the presence of other compounds, which have a slight antagonistic interaction with the Ah-receptor. The results of this part of the thesis suggest that the EROD bioassay could be developed to a very sensitive screening tool for cost conscious, high-through put breast milk-monitoring program.

Biotransformation of xenobiotics *in vivo* is usually part of a process of deactivation and elimination, but proestrogens (e.g. certain phytoestrogens and organochlorines) may undergo metabolic activation to potent estrogens in various body compartments. Therefore, the objective of the second part of this thesis was the investigation of P450 enzyme regulation in H4IIE cells for the metabolic activation of Bisphenol A as xenoestrogen model compound. This step was combined with recombinant yeast assay for the detection of the metabolites of this compound.

The experimental protocol was optimized and the results showed that most obtained metabolites of BPA formed are less active than the parent compound, suggesting the hypothesis that they represent conjugated fraction species, which are known for their weak estrogenic activity. This finding is in agreement with previous investigations with other metabolic activation systems based on primary cells.

1 Introduction

There is considerable public, regulatory, and scientific concern regarding human exposure to dioxin-like compounds. PCDD/F and PCB show similar properties and display a wide variety of toxic effects in mammals, birds, and fishes. Among the toxic effects observed because of exposure are immunotoxicity, carcinogenicity, metabolic changes, endocrine disruption, and even death.

Dioxin-like compounds have two types of their origin. PCB have been intentionally manufactured and PCDD/F have been occurred as unwanted by-products in chemicals or formed in thermal processes. These persistent toxic substances are now found in all kind of reservoirs, e.g. contaminated landfills, sediments/soils and tend to be accumulated in biota.

Seven polychlorinated dibenzo-p-dioxins (PCDD), 10 polychlorinated dibenzofurans (PCDF), and 12 polychlorinated biphenyls (PCB) are collectively referred to as dioxin-like compounds [1]. These compounds have been recently involved in several accidents, which led to possible contamination of feed/food products (e.g. chicken gate scandal, Belgium, [2] and citrus pulp pellets scandal in Brazil [3]). When dealing with such accidents or conducting monitoring, it would be useful to have screening methods, which can eliminate false positives prior to performance of chemical analysis. These assays should, rank the potency of substances and complex mixtures suspected of the contamination, are cost-effective, fast, have a minimum of false-negatives, and accepted by governmental regulators.

Until about 10 years ago, high-resolution gas chromatography/mass spectrometry (HRGC/HRMS) was the only option and became the "golden standard" for detecting dioxin-like compounds. Since 1970, it has been estimated that more than 1 billion US\$ has been spent on determining the toxicity of PCDD/F in samples [4]. Therefore, in the last decade, recent advances in the biotechnology have allowed to develop a battery of *in-vitro* bioassays and ligand binding assays for dioxin and dioxin-like compounds. By screening a large number of samples from a site, the most contaminated locations are identified, and there is a substantial cost saving when expensive analyses are not conducted in clean areas. This type of two-step process not only saves money, but also improves the accuracy, reliability, and scientific basis for the quantitative assessment of environmental health risks. For several bio-analytical dioxin tests, official methods by governmental authorities have now been

approved such as EPA Method 4425 (Reporter gene assay) or EPA Method 4025 (Immunoassay) [5].

Several experts have agreed in 1997 that the use of *in vitro* bioassays provides a useful tool as a pre-screening method for TEQs in environmental samples [6-8].

Furthermore, there are growing concerns regarding human health effects of dioxins and dioxin-like compounds to groups at higher risk for exposure as well as to the general population. Prospective cohort studies need to be established to evaluate risks of low-dose exposures over an extended period of time. For these studies, it is necessary to facilitate development of more simple, quicker, cheaper, and more precise measurements of dioxin body burden. To assess concentrations of dioxins and dioxin-like chemicals in human biological samples, blood may be the most practical specimen for an epidemiological study. However, the current chemical analysis method (HRGC/MS) requires collecting 50 mL to 100 mL of blood to detect all the congeners of dioxins and coPCB for WHO-TEQ calculation. The sampling volume is too much for ordinary volunteers in epidemiological studies. In occupational setting, welders, workers around coke ovens, kilns, and demolition site of municipal incinerators suffer from higher exposure to dioxins. However, occupational monitoring of dioxin exposure is not feasible considering the sample volume needed for HRGC/MS. Additionally, the measurement by HRGC/MS is expensive and requires several weeks, nevertheless, there is still an intrinsic limitation to the chemical analysis for describing the possible biological and toxicological effects of dioxin in animals. These conditions make it difficult to conduct large-scale epidemiological studies in the general population at higher risk.

Hence, the mechanism of action has been extensively studied over the past decades. The binding to the Ah receptor offers now several new technologies for the biochemical analyst to analyze the dioxin-like activity of these compounds quantitatively. Activation of aryl hydrocarbon receptor (AhR) and induction of CYP1A1 isozyme, whose activity can be determined by measuring the activity of the 7-ethoxyresorufin-o-deethylase (EROD) in various species and test systems, are well known bio-analyses for the assessment of toxicity of persistent toxic compounds.

Whereas analytical methods determine the concentrations of known substances, bio-analyses with EROD as an end point may also detect the joint activities of non analyzed EROD inducing compounds in environmental samples. The total sum of toxic equivalents (TEQ) of all kinds of dioxin-like compounds can be measured.

Advantages of these bioassays are the extremely high sensitivity, rapid, easy clean-up/work-up, and small sample size and reduced cost compared to instrumental methods. Bioassays have been already applied on a wide variety of matrices such as pure chemicals, food/feed, and environmental samples.

On the other hand, a number of chemicals released into the environment are believed to disrupt normal endocrine function in animals, thereby causing reproductive disorders and abnormalities in wildlife [9,10]. It has also been hypothesized that these chemicals are responsible for effects seen in humans, such as the concurrent increase in reproductive tract abnormalities and putative fall in sperm counts in men [11,12], and an increase in breast cancer in women. One major group of endocrine-disrupting chemicals, which could be responsible for these reproductive effects, is those that mimic natural estrogens: known as xenoestrogens. Of particular concern are the proestrogens, because the majority of the current *in-vitro* estrogenicity assays used to screen for suspect chemicals is likely to produce false-negative results for the prediction of estrogenic activity of such compounds *in vivo* due to a lack of their metabolic capability. Therefore, the EDSTAC recommended in its final report that, the evaluation of chemicals in the *in vitro* high throughput pre-screen (HTPS) should be performed in the presence and absence of metabolic activity to enhance the chances of detecting compounds with prohormonal activity. In addition, the ability to predict responses *in vivo* is questionable, as it is not possible to reproduce the *in vivo* pharmacokinetic and pharmacodynamic interactions in *in-vitro* assays. For example, *in vitro* assays do not possess the same metabolic capabilities present *in vivo* and therefore may generate false positive results due to the inability, to metabolically, inactivate an estrogenic substance. This has been observed with selected phthalate esters that were found to induce weak estrogen receptor-mediated effects *in vitro* but did not elicit a response *in vivo*, as evidenced by uterotrophic and vaginal cornification assays. Potentially more problematic are false negative results that are due to the inability of *in vitro* systems to bioactivate a proestrogen to its estrogenic metabolite. However, several *in vitro* systems possess some metabolic capabilities and, to date, there have been no reported examples of *in vitro* assays generating false negative results even with endocrine disruptors that are known to require bioactivation (i.e., methoxychlor, polychlorinated biphenyls). Thus, suitable test systems still need to be developed.

2 Objectives

Specific objectives of this thesis are:

- Screening of dioxin-like toxicity equivalents for various relevant matrices, with rat Hepatoma H4IIE cells bioassay (EROD) in biological matrices, environmental matrices and pure chemical.
- Screening approach for detection of novel dioxins sources and new dioxin-like compounds (novel Agonists): dioxin-like activity evaluation of new natural compound.
- Validation data of EROD bioassay for its application to biological human bio-monitoring studies, case of mother milk, and correlation with data from the analytical method GC/HRMS.
- Investigation of a possible presence of persistent estrogenic and dioxin-like compounds in leachate-polluted groundwater.
- Regulation of Cytochrome P450 in H4IIE for metabolic activation of Bisphenol A
- Development and optimization of a bioactivation protocol, combining metabolic activation with H4IIE cells and recombinant yeast assay, for screening the estrogenic activity of xenoestrogens after metabolism.

3. State of knowledge

3.1. The cytochrome P450 system

The cytochrome P450 enzyme system (the mixed-function oxygenases) is the major system for the metabolism of xenobiotic compounds, mainly for lipophilic compounds, such as drugs and organic pollutants. Mammals have at least 17 different families of cytochrome P450 (CYP) enzymes and about 50-60 genes coding for different enzymes. Families 1-4 are involved in xenobiotic metabolism, while the other families metabolise endogenous substrates or catalyse biosynthesis of endogenous compounds [13]. CYP enzymes are found in almost all mammalian tissues, but are present in highest levels in liver [14]. Thus, the liver is also the most studied organ, due to the high enzyme activity and relatively large organ size [15]. The central role of cytochrome P450 in detoxification, the specificity, and the extreme sensitivity makes it suitable for evaluations of pollution and exposure [15]. Cytochrome P450 enzymes can be induced by exposure to certain chemicals. The specificity for certain classes of chemicals makes it possible to use the increased P450-activity as a biomarker, to determine exposure to environmental contaminants. The first application of cytochrome P450s as biomarkers for contaminants was made on fish species [16], with later application on mammals and birds [17]. P450s have been well studied in mammals and Fish, but less in birds. It is stated that the lowest P450 activity is found in fish and fish-eating birds, intermediate activity in other bird species, and the highest activity in mammals [15]. A correlation between low hepatic cytochrome P450 activity and xenobiotic half-life have been demonstrated for vertebrates, and this is suggested to be the cause for the bioaccumulation of HAHs in some fish-eating birds [15].

3.1.1. Function and classification

The P450 system is located on the smooth endoplasmic reticulum (ER) in the cell. The system includes cytochrome P450 enzymes, the electron-transferring protein NADPH-cytochrome P450, and the embedding phospholipids of the ER. The cytochrome P450 are involved in the phase I reactions of biotransformation, mainly catalyzing oxidation of the substrate, or reduction under anaerobic conditions. The enzymes can also catalyze hydroxylation, epoxidation, dealkylation, and

desulfuration. The system activates xenobiotics in phase I reactions, by adding a polar groups, and allows substrate to conjugate in phase II reaction, so they become more lipophilic, and can be eliminated [14, 15].

3.1.2. Avian cytochrome P450

Historically, substances that induce the P450 system were divided into two classes, the methylcholantrene-type (MC-type) and the phenobarbital-type (PB-type), based upon their biochemical and morphological responses they exert in laboratory rodents. The MC-type inducers cause a slight increase in liver weight and cause induction of cytochrome P450 subfamily 1A, (cytochrome P448, e.g. AHH). The PB-type cause hepatic hypertrophy, proliferation of smooth ER increasing protein synthesis and induction of mammalian cytochrome P450 subfamily 2B. This group includes barbiturates, pesticides (e.g. DDT), and chemicals (e.g. ortho-PCB and -PBBs). However, this classing is inappropriate today, as the modern techniques has revealed a more complex patterns of enzymes induction [15].

Today the CYP enzymes are classified in families and subfamilies All enzymes with > 40% homology in amino acid sequences belong to the same family (e.g. CYP1, CYP2), and those with > 55% homology are further divided into subfamilies (e.g. CYP1A). Individual enzymes are numbered and designated e.g. (*CYP1A1* or *CYP1A2*), and the numbering order is the order in which they were discovered, independent of in which species it was found [14].

Humans have been estimated to have at least 53 different CYP genes and 24 pseudogenes [18]. The notable diversity of CYP enzymes has given rise to a systematic classification of individual forms into families and subfamilies. The protein sequences within a given gene family are at least 40% identical (e.g. CYP2A6 and CYP2B6), and the sequences within a given subfamily are > 55% identical (e.g. CYP2A6 and CYP2A7) [18]. The italicized names refer to genes, e.g. *CYP2A13*.

There are 17 different families currently known in humans. The enzymes in the families 1-3 are mostly active in the metabolism of xenobiotics, whereas the other families have important endogenous functions (Table 3.1). Inactivating mutations in the CYPs with physiological functions often lead to serious diseases, whereas similar mutations in xenobiotic-metabolizing CYPs rarely do, although they affect the hosts

drug metabolism and susceptibility to some diseases, without directly causing disease.

Table 3.1. Human CYP families and their main functions [18]

CYP family	Main functions
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism Arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid hydroxylation
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7-hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage Aldosterone synthesis
CYP17	Steroid 17-hydroxylation
CYP19	Androgen aromatization
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24-hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Steroid 27-hydroxylation
CYP39	Unknown
CYP46	Cholesterol 24-hydroxylation
CYP51	Sterol biosynthesis

3.1.3.1. AhR mechanism

While the PB-type inducers of cytochrome P450s appear to not be bound by a receptor, the MC-type inducers are bound in the cytosol to a receptor complex [15]. The majority of effects exerted by HAHs are thought to be mediated through the aryl hydrocarbon receptor (AhR) and the subsequent synthesis of certain proteins, including cytochrome CYP1A. The binding to the AhR, causes specific induction of CYP1A enzymes; two in mammals (CYP1A1 and 1A2), one in fish (CYP1A), and two in chicken (CP1A4 and 1A5) [19]. The induction pathway for the CYP1A subfamily is the best understood.

It is generally assumed that the substance acting as an AhR-ligand diffuses passively across the plasma membrane into the cytoplasm. Several studies however indicate that TCDD affects the cell membrane [20]. In the cytoplasm, the substance binds to the AhR-complex. Subsequently, the AhR complex is transformed releasing at least two heat shock proteins (hsp 90) and an AhR interacting protein (AIP). As the AhR-ligand complex translocates into the nucleus and associates with the AhR nuclear translocator protein (Arnt). Following a transformation into a complex with high DNA-affinity, it binds to the DNA responsive element (DER), is specific DNA recognition sequences, and gene transcription is induced [20, 21]. Once the HAH has bound to the receptor, it can not be replaced by another competing HAH [22]. At low HAH concentrations, a mixture of HAHs would give an additive effect, but at higher concentrations, the effect depends on the AhR affinity of the compounds. The activation of the AhR mechanism by HAHs, i.e. translocation to the nucleus, heterodimerization with Arnt and translocation of the active transcription factor to the DER binding site, is correlated to the AhR affinity of the HAH [22].

An alternate mechanism for the AhR-ligand induction has been suggested and it is not fully elucidated whether the AhR indeed is cytosolic. It has been suggested that the AhR might be situated in the nucleus and that formation of AhR -complex only enhances the DNA-affinity of the receptor, or alternatively, that the receptor is DNA-bound, and formation of the complex changes DNA-conformation [20].

The AhR has been identified in several human tissues and cell cultures, including lung, liver, kidney, placenta, and B-lymphocytes. In rats, the AhR has been identified

in thymus, lung, liver, kidney, brain, testis, and skeletal muscle, but not in pancreas. One explanation suggested that it has evolved for detoxification of combustion products like PAH (e.g. benzo[a]pyrene). A second hypothesis suggests that the AhR has an endogenous, at the time unknown, ligand. Although TCDD lacks affinity for steroid receptors, and steroid hormones lack affinity for the AhR, the AhR share many properties with the steroid hormone receptors, which has led to a suggestion that AhR is indeed a mutated steroid hormone [20]. Recently, it was proposed that AhR has a role in (chick) embryo morphogenesis, since it is expressed in all tissues through the development [23].

The AhR ligand-site is hydrophobic and fits planar, aromatic, non-polar ligands with maximal dimensions of 14 Å x 12 Å x 5 Å. In addition, ligand binding is dependent on electric and thermodynamic properties of the ligand induced [21]. However, high affinity binding has been reported for molecules with properties that deviate from these structural requirements [20]

3.1.3.2. Bioactivation

The cytochrome P450 enzymes, metabolize both endogenous and exogenous compounds (phase I and II reactions), generally increasing the water solubility of substrates, thereby enhancing their elimination. In this way, cytochromes P450 as CYP1A tend to detoxify xenobiotic chemicals. However, in some cases the substrate becomes more toxic after phase transformations catalysed by the cytochrome P450 enzymes than the parent compound. This can happen when the intermediate is resistant to further transformation into phase II conjugates. Some examples are the epoxidation of alrin to dieldrin, desulfuration of parathion to paraoxon, and the activation of some PAH and PCB to carcinogenic intermediates. The epoxidation results in formation of electrophilic arene oxides able induce single strand breaks on DNA [15, 24]. In addition, hydroxylated metabolites of PCB are demonstrated to bind covalently to macromolecules [24]. Whether CYP1A enzymes are harmful or protective, depend on the catalytic activities that they exert and the cells in which the enzyme is induced

3.2. Dioxin and dioxin-like compounds

Incomplete combustion of organic matter in combustion chambers leads to the formation of Halogenated aromatic compounds that have been widely dispersed in the environment. Many of these compounds are persistent, toxic, and bioaccumulate in food chains. Some toxic halogenated aromatics have a specific Ah receptor-mediated mechanism, for which structure activity relationships (SARs) have been derived. The most toxic halogenated aromatic is 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD or TCDD) and the toxicity of other individual halogenated aromatics have been determined relative to TCDD. This toxic equivalency factor (TEF) concept is used to determine a toxic equivalent concentration (TEQ) in a sample. A compound may be more or less dioxin-like. In this context dioxin-like refers to compounds that have similarities to TCDD in terms of structure, physicochemical properties, and in the biochemical and toxic responses that they elicit. It has been proposed [8, 25] that to include a compound in the TEF scheme it should:

- share certain structural relationships to the PCDD/F.
- bind to the Ah receptor.
- elicit Ah receptor-mediated biochemical and toxic responses.
- be persistent and accumulate in the food chain.

The three potential classes of compounds with dioxin-like properties that can bind to the AhR:

(A) Hydrophobic aromatic compounds with a planar structure and a size that the molecule or a part of the molecule fits into the binding side of the AhR. Examples include the planar congeners of PCB and PCDD/F; polychlorazobenzenes (PCABs), polychlorazoxybenzenes (PCAOBs), polychlorinated naphthalenes (PCNs), and several high molecular weight polyaromatic hydrocarbons (PAH).

(B) Other potential AhR agonists are compounds with a specific stereochemical configuration; such as polyhalogenated (chlorinated, brominated, fluorinated), mixed halogenated (chlorinated, brominated, fluorinated), and alkylated analogs of the previously listed class of compounds, chlorinated xanthenes and xanthenes (PCXE/PCXO), polychlorinated diphenyltoluenes (PCDPT), anisols (PCA), anthracenes (PCAN), fluorenes (PCFL), etc.

(C) Transient inducers and weak AhR ligands that deviate from the traditional criteria of planarity, aromaticity, and hydrophobicity and are rapidly degraded by the induced detoxification enzyme. This class includes natural compounds like indoles, heterocyclic amines, some pesticides, and drugs with various structures (imidazoles and pyridines).

3.2.1. Dioxin nomenclature and properties

Polychlorinated dibenzo-p-dioxin (PCDD) and furans (PCDF) are compounds with similar chemical properties. Each compound consists of two benzene rings interconnected by oxygen atoms (Fig. 3.1). Polychlorinated biphenyl (PCB) molecules are similar to PCDD and PCDF but a direct carbon bond without oxygen atom connects the two benzene rings. Polychlorinated benzene (PCBz) consists of only one benzene ring.

All PCDD and PCDF are organic solids with high melting points and low vapour pressure. They are characterized by extremely low water solubility, and have the ability for being strongly absorbed on the surface of the particulate matter. The water solubility of dioxin and furan decreases and the solubility in organic solvents and fats increase with increasing the chlorine content. Non- and mono-ortho-substituted PCB congeners have a high toxicity, similar to the polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-p-furans (PCDD/F). Therefore, these dioxins like PCB are referred to as dioxin-related compounds (Fig. 3.1). These congeners are strongly hydrophobic and thus highly lipophilic. [26]

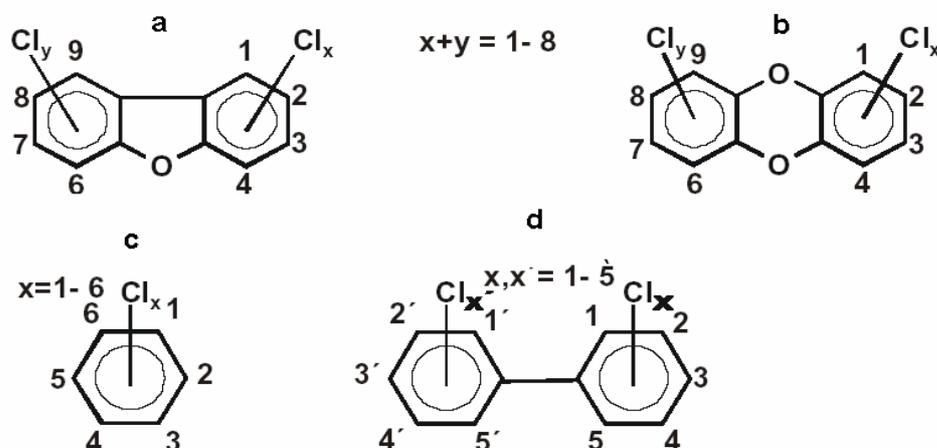


Fig. 3.1: Polychlorinated di-p-benzo-dioxin (a); Polychlorinated di-p-benzofuran (b); Polychlorinated benzene (c); Polychlorinated biphenyl (d)

There are 75 PCDD, 135 PCDF, 209 PCB, and 19 PCBz each differing in the number of the position of the chlorine atoms. Each individual PCDD or PCDF is referred to as a congener (giving 210 in total), while group of congeners with the same number of chlorine atoms are called homologues. The number of the congeners in each homologues group is shown in Tab. 3.2.

Table 3.2: Homologues and congeners of PCDD, PCD, PCB and PCBz

Homologue	Number of congeners			
	PCDD	PCDF	PCB	PCBz
Monochloro (M)	2	4	3	1
Dichloro (D)	10	16	12	3
Trichloro (Tr)	14	28	24	3
Tetrachloro (T)	22	38	42	3
Pentachloro (Pe)	14	28	46	1
Hexachloro (Hx)	10	16	42	1
Heptachloro (Hp)	2	4	24	-
Octachloro (O)	1	1	12	-
Nonachloro	-	-	3	-
Decachloro	-	-	1	-
Total	75	135	209	12

Often the term “dioxin” means PCDD and PCDF. PCDD and PCDF congeners with chlorine atoms in position 2, 3, 7, and 8 are of particular concern, especially the tetrachloro-CDD congener 2, 3, 7, 8-TCDD that is the most toxic dioxin. The toxicity effect of the dioxins present in the emission gases by the so-called “toxic equivalent” or Toxicity Equivalent Factor (TEF) is estimated. It is generally assumed that only 17

of the 210 dioxin and furan congeners and 12 of the 209 PCB congeners are toxic. Since 2,3,7,8-TCDD is the most toxic, its TEF is 1.0. The most toxic PCB congener among the 209 isomers is 3,3',4,4',5-PeCB. If the assigned value of the isomers is converted by the TEF, the final sum is so-called Toxic Equivalent (TEQ). TEQ is individual for each congener. The international values of the TEFs are termed into international toxic equivalent factor, or I-TEFs. The I-TEFs of the seventeen 2,3,7,8-positioned congeners of PCDD and PCDF are present in Tab.3.3. The sum of the individual TEQs for a mixture of PCDD and PCDF is termed the international toxic equivalent or I-TEQ.

$$\sum_{i=1}^n c_i * I-TEF_i = I-TEQ.$$

The World Health Organization (WHO) undertook the recent revision of the TEF scheme. The proposed scheme includes coplanar congeners of PCB by defining TEFs for 12 coplanar PCB. They are also listed in Table 3.3.

Table 3.3: Toxic equivalent factor (I-TEFs)

Congener	I-TEFs	WHO TEF
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	0.5	1
1,2,3,4,7,8-HxCDD	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01
OCDD	0.001	0.0001
2,3,7,8-TCDF	0.1	0.1
2,3,4,7,8-PeCDF	0.5	0.5
1,2,3,7,8-PeCDF	0.05	0.05
1,2,3,4,7,8-HxCDF	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01
OCDF	0.001	0.0001
3,4,4',5-TCB (#81)	-	0.0001
3,3',4,4'-TCB (#77)	-	0.0001
3,3',4,4',5 -PeCB (#126)	-	0.1
3,3',4,4',5,5'-HxCB (#169)	-	0.01
2,3,3',4,4'-PeCB (#105)	-	0.0001
2,3,4,4',5 -PeCB (#114)	-	0.0005
2,3',4,4',5-PeCB (#118)	-	0.0001
2',3,4,4',5-PeCB (#123)	-	0.0001
2,3,3',4,4',5-HxCB (#156)	-	0.0005
2,3,3',4,4', 5'-HxCB (#157)	-	0.0005
2,3',4,4',5,5'-HxCB (#167)	-	0.00001
2,3,3',4,4',5,5'-HpCB (#189)	-	0.0001

3.2.2. Uses and sources

Chlorinated dioxins and furans are not intentionally manufactured, rather, they are formed as by-products of fossil fuel and wood combustion; industrial, municipal, medical, and domestic waste incineration; metal smelting; and chlorine bleaching and manufacturing processes. Chlorinated dioxins and furans are also formed by combustion of polyvinyl chloride (PVC, commonly used in consumer product packaging and medical devices), and polychlorinated biphenyls (PCB). Uncontrolled combustion processes, such as fires in buildings, can lead to formation of both chlorinated and brominated dioxins and furans. Polybrominated dioxins and dibenzofurans are formed when flame-retardants used in certain plastics (e.g., textiles and foam furniture cushions) are burned. The Stockholm Convention will require governments to take steps to minimize and, where feasible, eliminate releases of dioxins and furans.

3.2.3. Routes of human exposure

In the environment, dioxins and furans are found in soil, sediment, and food because of industrial and combustion processes.

Once in the environment, these compounds can remain for years and even decades before breaking down through natural processes. Dioxins accumulate in the fat of food-producing animals and are transferred into meat, eggs, and dairy products. EPA estimates that most (95%) dioxin exposure occurs through eating these animal fats (Figure 3.3). People who eat higher than average amounts of contaminated foods (e.g. sport or subsistence fishermen) can be exposed to significantly higher amounts of dioxins (as well as other persistent chemicals) than the general public. Once in the human body, dioxins can be stored in fatty tissues for long periods. They can be released into human breast milk, leading to exposure to nursing infants. Despite the presence of dioxin in breast milk, medical experts still strongly support breastfeeding, based on its clear health benefits for infants. Additional study is needed to evaluate long-term effects of exposure to dioxin and other persistent chemicals in breast milk. Other populations that may have dioxin exposures higher than the general population include cigarette smokers and people living near incinerators. In addition to food,

people are exposed to dioxins and furans through contact with contaminated soils, urban air, and water sources, although the levels of contamination found in these sources are typically much lower [27].

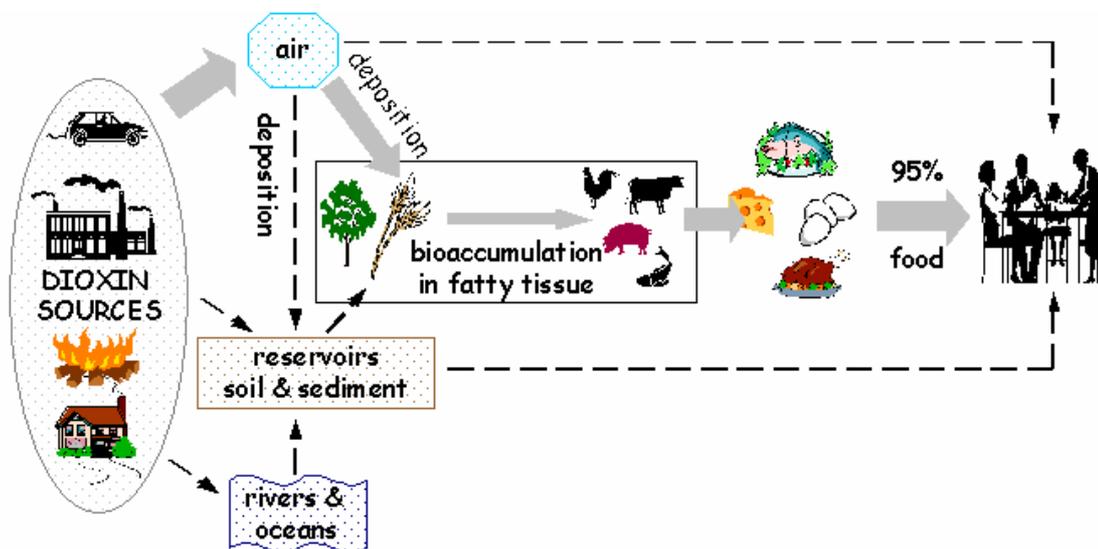


Fig. 3.2: Sources and pathways of dioxins (Fact sheet)

3.3. *in vitro* bio-analyses for dioxin-like compounds screening

Toxicity assessment of environmental samples is difficult, as there are hundreds of compounds, isomers and congeners that differ in toxic potency. Risk assessment of specific halogenated aromatic (HAH) congeners require information about identify and characteristics specific for that molecule. Analytically, appropriate standards have to be available and in the case of mixtures, appropriate separation methods are needed. The use of analytical data in risk assessment is limited if relative potencies and interactive effects are not fully elucidated [24, 28, 29].

Environmental samples often contain a very complex mixture of substances. The toxic potency of mixtures can be estimated by assuming additive effects. For this propose the Toxic Equivalent (TEQ) has been developed, by this approach assessment of toxicity in a mixture can be made to some extent [8]. However, the assumption of additive effects when calculating TEQ-values neglects the fact that synergistic and/or antagonistic effects may occur. Also, the TEFs mostly used are derived from mammalian toxicity studies, which is not suitable for toxicity assessment for certain species and the endpoints [28, 29]. Quantification of all possible active

compounds in a sample is time consuming and expensive, and practically impossible. Therefore, bioassay analysis of dioxin-like compounds is suitable for complex samples. The bioassay measures dioxin-like activity and accounts for potencies and interactive effects of known and unknown compounds [30]. Many studies have shown that bioassay derived TEQ values for complex samples are higher than those calculated from chemical analysis for the same sample. Thus it is suggested that other compounds than those routinely screened contributes to a relevant part of the total TEQ concentration. [29, 31, 32, 33]. Therefore, in a near future, there might be other dioxin-like compounds such as polychlorinated naphthalenes (PCNs), alkyl-polychlorodibenzofurans (R-PCDFs), and polychlorinated dibenzothiophenes (PCDTs) added to this list of toxic dioxin-like compounds

In vitro and *in vivo* evaluation of HAH toxicity correlate very well [24]. Thus, in the field of HAHs, several bioassays have been developed, and most of them are based on measure of P450 enzyme induced by HAHs. One advantage of these bioassays is the biological (i. e. mechanistic) specificity [29]. The induction mechanism via binding to the AhR receptors is strongly dependent on the molecular structure. The bioassays can be based on native biochemical responses or on artificially constructed responses involving reporter genes [29]. Several cell types from different organisms are commonly used in bioassay for analysis of HAHs, e.g. chick hepatocytes, rat hepatoma H4IIE, rainbow trout hepatocytes RTL-W1, and human HepG2.

In *in vitro* bioassays, metabolism and respiration of the cells are included in the response, as well as the mechanism of receptor binding and gene transcription. Thus, there is an interest to develop bioassay derived TEFs and TEQ concentrations that integrate important biological interaction [30, 34]. It should however be emphasized that, endpoints and exposure conditions *in vitro* are quite different from those *in vivo*, and one should be careful, when extrapolating data [29].

3.3.1. Background of EROD bioassay

In this work, the used bio-analytical method is Micro-EROD (Ethoxyresorufin-O-dealkylase assay) bioassay. The used continuous cell line, H4IIE, was derived from Reuber Hepatoma H-35 [35] by Pitot and coworkers [36]. A decade later induction of CYP1A catalytic activity in the cell line was demonstrated [37, 38]. The H4IIE cells

are well suited for the examination of EROD induction due to their excellent growth characteristics and the presence of low basal, but highly inducible, CYP1A activity. The H4IIE cell lines are exquisitely responsive to 2,3,7,8-TCDD –based CYP1A induction. These characteristics prompted researchers from the U.S. Food and Drug Administration to develop and characterize a contaminant detection bioassay based on the H4IIE cell line [39]. This original chemical in foodstuffs as indicated by aryl hydrocarbon hydroxylase (AHH) activity, a catalytic measure of CYP1A [40]. The assay was subsequently modified to examine EROD activity rather than AHH activity [41] because the EROD assay employs a non-toxic substrate.

The induction of CYP1A is mediated through the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR) (Fig 3.3). AhR ligands generally have isomeric configuration and are similar in structure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), a model CYP1A inducer. Receptor binding is followed by a series of molecular events leading to the expression of several genes (including CYP1A) known as the “Ah-gene battery”.

Use of the H4IIE assay to rank the toxic potency of individual chemicals based on the 2,3,7,8-TCDD equivalency (TEQ). TEQ values generated by the H4IIE assay provided relative toxicity estimation for individual chemicals. The values can also be used together with analytical chemistry to evaluate the potential interactions of mixtures of CYP1A-inducing chemicals in biological systems. More recently, environmental assessments using the H4IIE bioassay have become more prevalent, mainly due to the systematic characterization of the assay by Tillitt [42]. Additional modifications that have improved the bioassay have also been introduced over the past decades [43-47]. This Bioassay is largely and successfully used for analysis of different complex matrix, for example Emissions control [48].

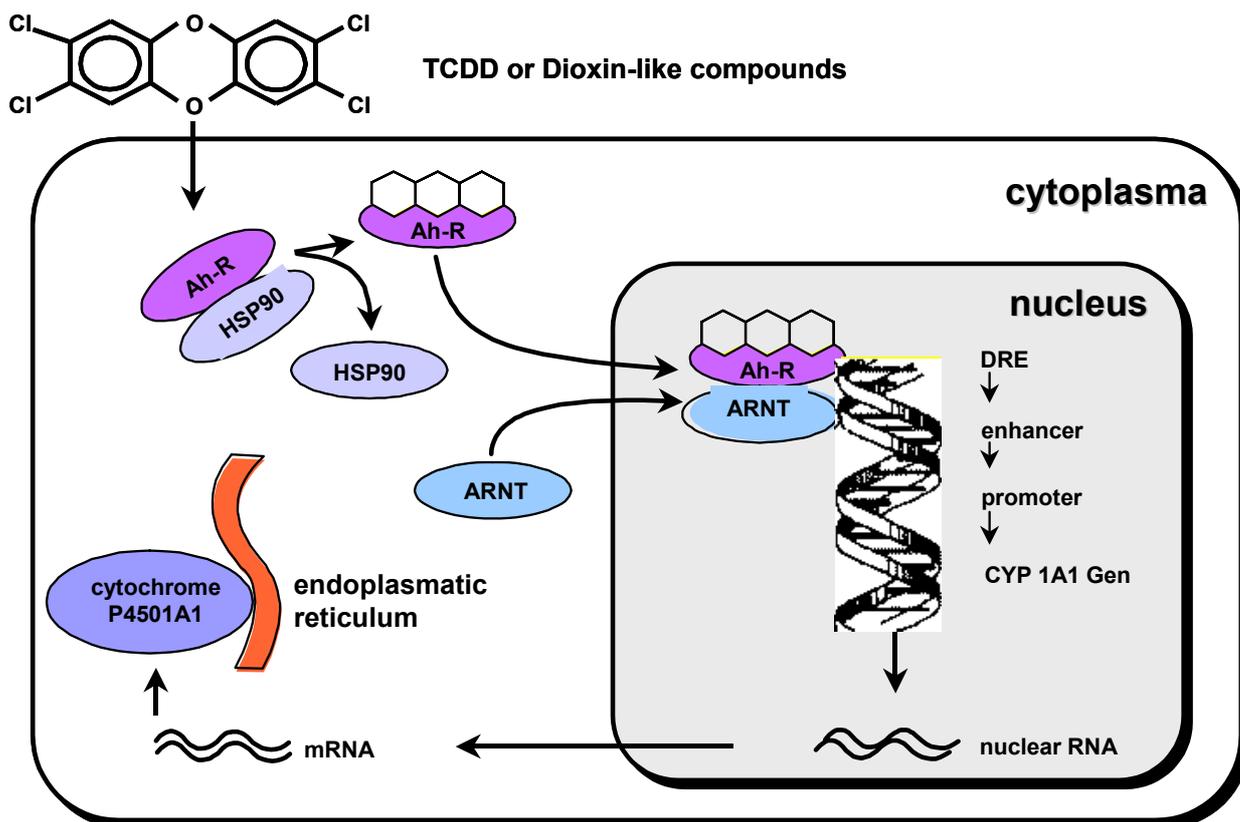


Fig. 3.3: Proposed mechanism of AhR-mediated toxicity in EROD bioassay

3.3.2. Validation and quality in bioassays

The bio-analytical methods for dioxins and dioxin-like compounds screening are being world wide used and developed in many laboratories, but this bioassays system still has not been subjected to a formal validation study for bio-monitoring investigations.

The aim of a test method validation of is, to demonstrate that the method is fit for the intended purpose and that the uncertainty in the results is acceptable. However, it is important that the rules of the validation should not prevent natural technological development from taking place. If a method is widely used, a collaborative study involving a group of laboratories (inter-laboratory comparisons) can be organized for validating a method.

Validation includes all the procedures demonstrating, that a particular method used for quantitative analyze in given matrix is reliable and reproducible for the given task. If published methods are modified to suit the requirement of the laboratory-

performing assay, these modifications should be validated. Different types and levels of validation are defined and characterized as follows [49]

- When developing and implementing a bio-analytical method for the first time or for a new toxic compound, a full validation is needed.
- For modifications of bio-analytical methods, which typical modifications can be a change in the matrix, in the test organism, in the principal of the test (detection system), in instruments and/or software or laboratory or when quality controls indicate that an established method is changing with time, a partial validation is required.

For demonstration of the equivalence between two or more bio-analytical methods (for example, comparison of a reference bio-analytical method (already validated) with a new bio-analytical method or a comparison of data generated using different analytical techniques vs. bioassay, a cross-validation can be carried.

In this work, a cross validation of EROD micro assay was performed, by participation in an intercalibration study, with other known bioassay for dioxin-like compounds detection, and the data are compared vs. the standard analytical method GC/MS for different matrix with different level of contamination.

3.4. Relevant environmental matrices for EROD application

3.4.1. Biological matrix: Human milk

Breast milk has been widely used in bio-monitoring programs to assess human exposure to dioxins and dioxin-like compounds. The WHO European Centre for Environment and Health has conducted several studies in breast milk in countries worldwide, designed to assess levels and changes in level of polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDFs) and certain polychlorinated biphenyls (PCB) known to have “dioxin-like” properties.

Although breast-feeding women cannot be representative of the general population. For bio-monitoring programs carried out to assist in policy designed to improve public health and safety, it is important to monitor dioxin exposure of this demographic segment through breast milk due to several reasons:

Breast milk reflects the maternal body burden of lipophilic chemicals and thus it is a measure of prenatal exposure to those compounds, being a human food and the first and main foodstuff for most newborn babies during first lifetime-period, breast milk can be a very significant pathway for infant exposure to dioxins

Because large volumes can be collected non-invasively, breast milk is also a convenient sampling specimen for bio-monitoring purposes if it is collected taking into consideration all the relevant factors influencing fat content and thus levels of lipophilic compounds, namely the time of sampling during lactation, breast-feeding patterns and maternal characteristics.

It is a general consensus that human milk is exclusively excellent for infant feeding, providing health and growth advantages for infants [50-52]. However, a considerable number of studies have reported, that the contamination of breast milk with organochlorine hydrocarbons may pose adverse health effects for the offspring. It was reported, that breast-feeding was the major route of PCB and dioxin exposure for infants, with the average daily intake of polychlorinated dibenzo-p-dioxin (PCDD) (about 100 pg TEQ/kg body wt. at age 2 months) being about 50-fold higher than that for formula-fed babies [53]. In Japanese and Taiwanese rice oil incidents, the children of women accidentally exposed to high dietary intake of PCB and related compounds experienced a variety of adverse health effects (e.g., low birth rate, hyper pigmentation, and neurological, cognitive, developmental, and behavioral abnormalities [54-57].

Dioxin was first detected in human milk samples from Germany in 1984, followed soon thereafter by confirmation of comparable levels of contamination in other industrialized countries [27].

Moreover, Studies on PCB and pesticides levels in breast milk have been conducted world wide in the past 30–40 years. However, a little investigation reported dioxin levels in breast milk; this was because the measurement of dioxin is very expensive and time-consuming, particularly for a routine and high-throughput breast milk-monitoring program. Hence, numerous studies have tried to develop a more cost-efficient assessment of dioxin exposure. Most studies have adopted the use of PCB congeners in breast milk as markers for dioxin measurement [58,59]. However, the association between concentrations of PCB markers and dioxin may vary among

individual study populations with different geographical and cultural backgrounds. The underestimation of actual dioxin concentrations using PCB markers has been reported [58,60]. Nevertheless, there is still an intrinsic limitation to the chemical analysis for describing the possible biological and toxicological effects of dioxin in animals.

3.4.2. Environmental matrix: leachate-polluted groundwater

Groundwater contaminated with landfill leachate presents a very complex mixture that may contain a large number of xenobiotic organic compounds. These compounds are usually found in low concentrations ($\mu\text{g/L}$), but all the compounds in combination may cause severe biological effects, as many of the identified compounds are highly toxic or even carcinogenic. At unlined landfills, the compounds may leach directly to the groundwater. This poses serious risks for ecosystems and human health if the groundwater reaches surface water or is used as a drinking water supply. The potential risks associated with xenobiotic organic compounds in groundwater contaminated with landfill leachates have been pointed out by several authors [61-63], but the actual toxicity of the contaminant mixture has received very little attention.

Chemical identification of xenobiotic organic pollutants in groundwater provides valuable information for hazard assessment, but chemical identification alone does not reveal factors such as toxicity, possible toxic interactions, or toxic degradation products of the compounds present in leachate-polluted groundwater. By contrast to chemical analysis, a bioassay approach for toxicity characterization of environmental samples integrates the biological effect of all compounds present. Thus, factors such as bioavailability, synergism, or antagonism can be assessed directly without the need for identification of each contaminant classes and estimation of individual compound toxicity. Only a few studies have assessed the toxicity of landfill leachate using bioassays, and even fewer have dealt with the monitoring of dioxin-like and estrogenic activity of persistence compound in leachate-polluted groundwater near landfills.

It was reported that, the dioxin-like activity of leachate-polluted may be attributed generally to the presence of dissolved chlorinated compounds: polycyclic aromatic hydrocarbons, to the low molecular weight compounds, Benzofuran, Indane and

Indene [64]. Furthermore, dioxins have a low solubility in water (e.g. 19.3 ng/L for 2,3,7,8-TCDD) but their sorption from leachate onto organic matter may give rise to the facilitated transport of these compounds into ground water [65]. This is a particular concern complexation of hydrophobic chemicals with organic matter can also inhibit the ability of micro-organisms to degrade these compounds even though they may still be available and therefore toxic to higher organisms.

3.4.3. Natural halogenated organic compounds

Despite recent efforts with “classic anthropogenic” contaminants (DDT and PCB among others), new emerging organohalogen compounds of diverse structure are currently under discussion [66]. Most of these organohalogens are persistent, bioaccumulative, and might thus be a long-lived problem. It has long been thought, that the adverse properties discussed so far are exclusively valid for man-made substances. Thousands of naturally produced organohalogens are known [67-69] [70]; these halogenated natural products were thought not to be persistent. Recently, however, halogenated natural products have been detected at high concentrations in marine biota [71-75], and structural similarities between hazardous anthropogenic pollutants and these natural products have been demonstrated [71,73,76]. One of the marine halogenated natural products of concern is Q1, this compound was detected at high concentrations (up to 14 ppm) in a wide range of biological samples [77], which most likely received contamination from uptake via food items. Synthesis and structure elucidation demonstrated that the structure of Q1 is 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole [78]. Recent evaluation clarified that Q1 is identical with a previously unknown compound detected in biological samples for 20 years. Initial measurements indicated a bioaccumulative behavior of Q1 in some species. In addition, the Log Kow was both calculated and experimentally GC-estimated to be 5.9-6.4. These observations in environmental samples along with initial determination of properties pointed towards Q1 being a compound that may be a representative of both the persistent organic pollutant (POP), and the persistent bioaccumulative and toxic compounds (PBT) concepts.

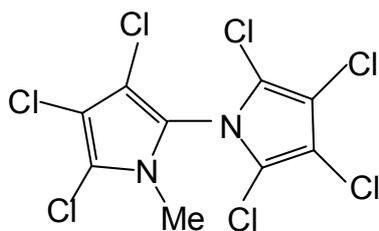


Fig. 3.4: Chemical structure of Q1

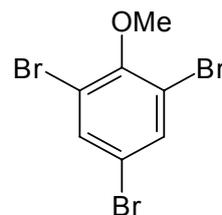


Fig. 3.5: Chemical Structure of TBA

3.5. Endocrine disrupters

There is considerable concern within the scientific community and media that the increasing occurrence of endocrine-related abnormalities in humans and wildlife may be associated with exposure to environmental pollutants capable of mimicking or modulating the action of natural hormones. Appreciation that many ubiquitous compounds of natural and anthropogenic origins are estrogenic has led to the hypothesis that exposure to such compounds may be involved in falling sperm counts and disorders of the male reproductive tract [79]. Although such estrogenic compounds are structurally heterogeneous [80], there are similarities including lipophilicity that facilitate accumulation in food-producing animals and increase risks associated with entry into the human food chain [81]. In the United Kingdom these concerns have been given added impetus following a report by the Institute for Environment and Health [82], which made several recommendations aimed at investigating the risks of hormonal mimics; these included the need to develop robust and reliable assays capable of screening chemicals for estrogenic activity. Given that prediction of estrogenic potency through structural information alone is not yet possible, development of such generic assays is necessary because many existing assay systems are not sufficiently robust for non-expert operation or may not accurately reflect human potency.

3.5.1. Identification and Assessment of endocrine disruptors

Much attention is being devoted to the development of *in vivo* and *in vitro* screening strategies to identify and classify xenoestrogens, in order to determine whether such chemicals pose a hazard to human health. Currently, different *in vivo* and *in vitro*

assays have been used to identify estrogenic activity of compounds or a mixture [83-85]. Moreover, much work has been done trying to establish structure–activity relationships, but the precise structural requirements for estrogenic activity are not yet fully understood. Therefore, a set of criteria is needed to determine the estrogenic activity of chemicals, to enable those that are capable of endocrine disruption to be identified.

The most widely used *in vivo* estrogen assay, the rodent uterotrophic assay, relies on the ability of chemicals to stimulate uterine growth [85-86]. The advantage of this assay is that it incorporates all aspects of distribution, and excretion of the chemical, and also for alternative pathways of endocrine disruption. However, even with this gold-standard assay, inconsistencies in results occur, depending on the route of administration and the response that is monitored [87]. Additionally, *in vivo* assays are costly and time-consuming, and therefore need to be used in conjunction with one or more reliable *in vitro* tests. The *in vitro* tests currently used range from simple competitive binding assays, relying solely on the chemical's ability to bind to the estrogen receptor [83, 86], to more complex systems where the chemical binds to, and activates, the receptor. These latter assay systems include the proliferation of the human breast cancer cell line (MCF-7) [84,88], vitellogenin gene expression in hepatocyte cultures [89], and yeast based assays expressing either rainbow trout [90] or human estrogen receptors [91,92]. However, *in vitro* assays do not always reliably predict the outcome of *in vivo* assays, since chemicals can be metabolically activated or inactivated *in vivo*, and may act independently with the receptor.

The inconsistent results between different *in vitro* systems [86,90], may also be partially due to the differing metabolic capabilities of the test systems used. Thus, whether a chemical is, or is not, identified as being estrogenic may depend on the actual test system used, and this calls for confirmation of any positive findings using other assays.

Thus, suitable test systems do exist, but methods still need to be validated and standardized. An *in vivo*–*in vitro* strategy is urgently needed, and indeed the U.S. Environmental Protection Agency is currently developing a chemical screening and testing program for endocrine effects. Additionally, there is a need for agreement concerning the boundaries within a biological response leads to a chemical being labeled as “estrogenic.”

The capacity of an organism to metabolize a compound to more polar products is often considered a detoxification mechanism. Furthermore, the presence of polar hydroxy groups in parent compounds often prevents bioaccumulation. In addition, introduction of even more polar groups such as glucuronides or sulfates further increases the ability of an organism to eliminate the compound.

A disadvantage of this approach is that the presence of hydroxy groups and complicated structures with aromatic moieties may bear resemblance to steroid structures. As a result, less bioaccumulative compounds such as alkylphenols, phthalate esters, and methoxychlor have shown biological activities similar to estrogens or androgens. These compounds can act as either (partial) agonists or antagonists for steroid receptors such as the estrogen receptor. Such interactions may have consequences for (sexual) development, reproduction, and the formation of hormone-dependent tumors.

Thus, subtle differences among molecules, such as the presence or absence of an OH group, can lead to significant changes in their ability to bind to steroid receptors or inhibit steroidogenic enzymes. The presence of an OH and/or aromatic group plays a significant role in both steroid metabolism and receptor binding [3,4]. Thus, biotransformation plays a significant part in the endocrine-disrupting properties of a compound. On the one hand, the introduction of an OH group may bioactivate the parent compound by forming a metabolite that can interact with a steroid receptor or steroidogenic enzyme: On the other hand, a rapid phase II metabolism producing glucuronides or sulfates helps the organism to eliminate the parent compound from the body, reducing the opportunity for adverse effects.

Methods of detecting and assessing estrogenicity of such compound have been previously described [93] and critically evaluated [94]. Biotransformation and consequent alteration of hormonal activity by test systems is an important consideration because phenolic metabolites produced by the action of cytochrome P450 enzymes can be more potent than the parent compounds [65,95]. Clearly, although such metabolic effects are intrinsic to *in vivo* models, they may not be so readily reflected by *in vitro* test systems. Estrogen-sensitive human breast cancer cells, such as MCF-7 cells, express the human estrogen receptor and have been used to develop E-screen tests for chemicals [96] and contaminants in animal feeds [97]. Uterotrophic assays, such as the classical mouse uterine weight bioassay [98],

have limitations [94], but are still preferred methods for many investigators [99]. Uterotrophic assays also offer the potential to combine biochemical and histological analysis of estrogen-mediated events and further adaptation to measure anti-uterotrophic activity [100].

The use of receptors linked to reporter genes in transformed cellular systems to detect biologically active xenobiotics has been proposed as a strategy to define chemicals by their functional properties [80]. This approach facilitates detection of not only receptor ligand binding but also response element occupancy and gene activation. The recombinant yeast cells used in the present thesis have been used in various estrogen receptor studies [101-104]. The particular advantage of the transformed yeast cell line approach is that the cells are robust and substrates auxotrophy may be used to continuously select for estrogen sensitivity. Moreover, expression of human estrogen receptor and ability to automate suggest that these yeast cells have much to offer the analyst requiring an *in vitro* screening assay that affords some reflection of potential estrogenic activity in humans.

3.6. Bisphenol A as xenoestrogen model

3.6.1. Production

Many countries throughout the world have large production capacities for BPA, especially Germany, the Netherlands, the USA, and Japan. Major companies include Dow, Bayer, Shell, GE Plastics, Aristech, Mitsubishi, Mitsui, and Shin Nihon.

In the EU alone, in 1997/98, annual consumption of BPA was estimated at approximately 640,000 tones (640 x 10⁶kg) per year. EU manufacturers of BPA include Bayer in Belgium and Germany; Dow in Germany; GE Plastics in the Netherlands and Spain; and Shell in the Netherlands. Global production is reported to be increasing at about 7% per year, and to meet the increase in demand, Bayer is opening a new factory in Thailand. However, in 1999, Shell Chemical's global BPA business was up for sale [105].

3.6.2. Uses

About 65% of the bisphenol A produced is used to make polycarbonate, and approximately 25% is used in epoxy resin production. The remaining 10% is used in other products such as specialty resins and in the manufacture of flame retardants, such as tetrabromobisphenol A [105]. Bisphenol A is therefore used in the manufacture of a great variety of products including: compact disks, food can linings, thermal (fax) paper, safety helmets, bullet resistant laminate, plastic windows, car parts, adhesives, protective coatings, powder paints, polycarbonate bottles and containers (including returnable milk and water bottles) and the sheathing of electrical and electronic parts. BPA is also used in PVC production and processing, where it may be used as a reaction inhibitor, and as an anti-oxidant [105] (Table 3.4).

3.6.3. Human exposure

3.6.3.1. Polycarbonate bottles

In 1993, a study by Krishnan and colleagues at Stanford Medical School found that heating polycarbonate laboratory flasks at 121°C (250 °F) for 25 minutes released 2-5 µg/kg of bisphenol A into water-filled flasks [105].

Some subsequent studies have not found any measurable amounts of BPA leaching from babies' bottles or test discs made of polycarbonate, but other studies in Japan and England have suggested quite significant leaching particularly from older scratched bottles. Studies which have found no leaching of BPA from babies bottles or polycarbonate test discs, include, for example, one by the Society of the Plastics Industry (with a detection limit of 5 µg/kg) and one by the UK Ministry of Agriculture, Fisheries and Food (with a detection limit of 30 µg/kg of liquid) [106]. Biles and co-workers also found that when whole polycarbonate bottles were tested with typical fill conditions using normal use conditions no migration was detected, although some migration was noted under exaggerated conditions. However, some studies in Japan have suggested that more BPA can leach from polycarbonate that has been scratched or is more than four years old. For example, in tests conducted at Nagasaki University in 1998, Koji Arizono and co-workers showed, that up to 6.5 µg/kg leached from old polycarbonate baby bottles heated up to 95°C for 30 minutes, but new bottles only leached up to 3.5 µg/kg (ppb). Arizono also found that scratched bottles from the Philippines leached approximately 30 µg/kg (ppb) of BPA and those

from Korea leached over 15µg/kg, more than 5 times the amount leached by new bottles [107].

3.6.3.2. Dental exposure

Some (but not all) dental resins contain bisphenol A [108] found, that a sealant containing bisphenol A diglycidylether methacrylate (bis-GMA) was oestrogenic to MCF7 breast cancer cells. Samples of the saliva from 11 patients taken 1 hour after dental treatment contained bisphenol A and bis-GMA. There is some dispute about the details of this research [109,111].

3.6.3.3. Alternatives

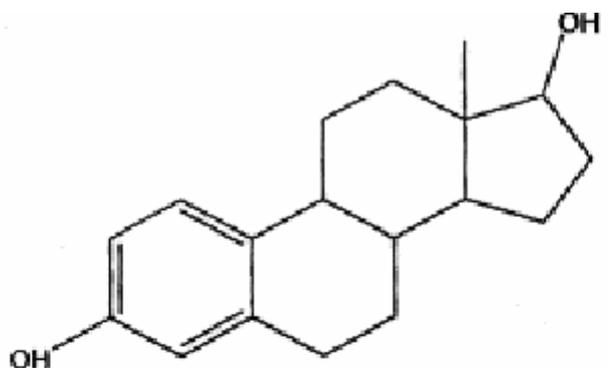
Bisphenol A is not used in all can lacquers, but the Metal Packaging Manufacturers Association considers that the industry might have problems switching to new formulations [112]. The general secrecy surrounding the chemicals used in can linings makes it very hard for any external observer to evaluate what alternatives are available.

Table 3.4: global bisphenol A capacity in thousand tones per year

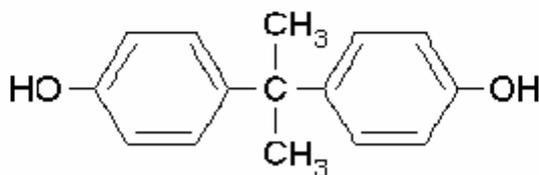
West Europe		thousand tones / year
Bayer	Antwerp, Belgium	140
Dow	Stade, Germany	100
	Krefeld-Uerdingen, Germany	160
Dow	Stade, Germany	100
GE Plastics	Bergen op Zoom, Netherlands	210
	Cartagena, Spain	110
Shell	Pernis, Netherlands	110
East Europe		
Petro Borzesti	Borzesti, Romania	10
ZC	Blachownia, Poland	10
North America		
Aristech	Haverhill, Ohio	110
Bayer	Baytown, Texas	120
		160
Dow	Freeport, Texas	45
		166
GE Plastics	Burkville, Alabama	68
	Mount Vernon	260
Shell	Deer Park, Texas	102
		113
Asia		
Wuxi Resin	Wuxi, China	10
Kesar	Loteparhram, India	7.5
Idemitsu	Chiba, Japan	70
Mitsubishi	Kashima, Japan	80
Mitsui	Nagoya, Japan	80
	Kyusu, Japan	95
Shin Nihon (Mitsubishi Chemical/Nippon Steel Chemical)	Osaka, Japan	60
Mitsui	Pulau Sakra, Singapore	70
Kumho P & B	Yeochon, S Korea	30
Nan Ya	Mailiao, Taiwan	72
Chang Chun	Mailiao, Taiwan	20
Taiwan Prosperity	Linyuan, Taiwan	25

3.6.4. Estrogenicity of Bisphenol A

Bisphenol A [BPA, 2,2-bis(4-hydroxyphenyl)propane; have a structure distinct from that of 17 β -estradiol (E2) (Fig. 3.6), its ability to bind to the estrogen receptor (ER) might be rationalized if the two phenol rings mimicked the A- and D-rings of E2, within the ligand binding domain of ER [113].



17- β - Estradiol



Bisphenol A

Fig. 3.6. Structure of 17- β -Estradiol and Bisphenol A

The estrogenic activity of BPA has been assessed by a variety of *in vitro* assays, including ER binding [114], yeast reporter-gene expression assays [115], proliferation of MCF-7 human breast cancer cells [116], and induction of progesterone receptors in both human MCF-7 cells [117] and endometrial carcinoma cells [118]. Studies *in vivo* have shown that BPA can mimic E2, in stimulating prolactin secretion [119], inducing growth, differentiation, c-fos gene expression in the female rat reproductive tract [120] and exhibiting uterotrophic activity in both rats [121,122] and mice [123]. There is concern, that the estrogenicity of BPA may elicit toxicity to mammalian developmental and reproductive processes. BPA can effect early development of

preimplantation mouse embryos [124], in addition to increasing prostate size as a consequence of low-dose fetal exposure [125]. Exposure to environmentally relevant doses of BPA has been shown to advance puberty and alter postnatal growth rate in mice [126]. In contrast, other studies using the same levels of fetal BPA exposure and the same mouse strain as Nagel et al [125] did not observe any effect on the prostate gland [127-128]. Also, male offspring from pregnant Wistar rats exposed to BPA in drinking water had normal reproductive organ development [62].

Metabolism can play an important role in modulating the estrogenic activity of xenoestrogens *in vivo* [129]. The metabolism of BPA has been well characterized in the rat with the major metabolite being the monoglucuronide (BPA glucuronide). BPA glucuronide constituted approximately 28% of the radioactivity found in urine and 68 to 100% of the plasma radioactivity of C14 labeled BPA. Glucuronidation of BPA by rat liver microsomes is mainly catalyzed by the UDP-glucuronosyltransferase (UGT) isoform UGT2B1 [166], Knaak and Sullivan also identified 5-hydroxybisphenol A [5-OHBPA, 2-(4,5-dihydroxyphenyl)-2-(4-hydroxyphenyl)propane], which has been postulated to be formed by rat liver microsomes [130].

Because the liver is the first barrier of exogenous drugs, many studies of drug metabolism in whole cells have been performed in isolated hepatocytes in culture, and perfused livers. Like most cell in culture, hepatocytes in culture undergo marked changes in enzymatic composition including cytochrome P-450. These techniques have their own particular advantages and disadvantages, isolated hepatocytes have been successfully in a number of studies of drug metabolism, they may be separated rapidly into cytosol and mitochondria, a distinct advantage over the perfused liver. However, isolated hepatocytes are fragile, because they not exist in their natural environment, and may deteriorate rapidly *in vitro* as indicated by a marked depletion of intracellular potassium [131]. In contrary, the continuous H4IIE hepatoma cell lines present many advantages, stability, excellent growth characteristics, the presence of low basal, but highly inducible CYP1A activity and they are somewhat easier to use than primary cell culture. Therefore, we have proposed in this study to use H4IIE hepatoma cells as a metabolizing tool of bisphenol A to investigate the estrogenic activity modulation after biotransformation by recombinant yeast assay.

3.7. Recombinant yeast estrogenicity assay: Background

A recombinant yeast strain was developed in the Genetics Department at Glaxo for use in a test to identify compounds, which can interact with the human estrogen receptor (hER). This assay allows for the generation of reproducible empirical biological data to be obtained in a cost-and-time-effective manner. A simple color change from yellow to red, measured by a spectrophotometer, indicated the presence of an estrogenic chemical. The intensity of the color change is directly related to the estrogenic activity. This assay has been shown to be suitable for both neat chemicals and environmental samples and complements the *in vivo* mammalian methods

Yeast cells do not normally contain an estrogen receptor; therefore, the DNA sequence of hER was stably integrated into the main chromosome of the yeast. The yeast cells also contain expression plasmids carrying the reporter gene lac-Z (encoding the enzyme β -galactosidase), which is used to measure the receptors' activity.

In this system, hER is expressed in a form capable of binding to estrogen-responsive sequences (ERE). These sequences were situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the estrogen-occupied receptor interacts with transcription factors and other transcriptional components to modulate gene transcription. This causes expression of the reporter gene lac-Z and the enzyme produced (β -galactosidase) is secreted into the medium, where it metabolizes the chromogenic substrate, chlorophenol red- β -D - galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorption at 540 nm.

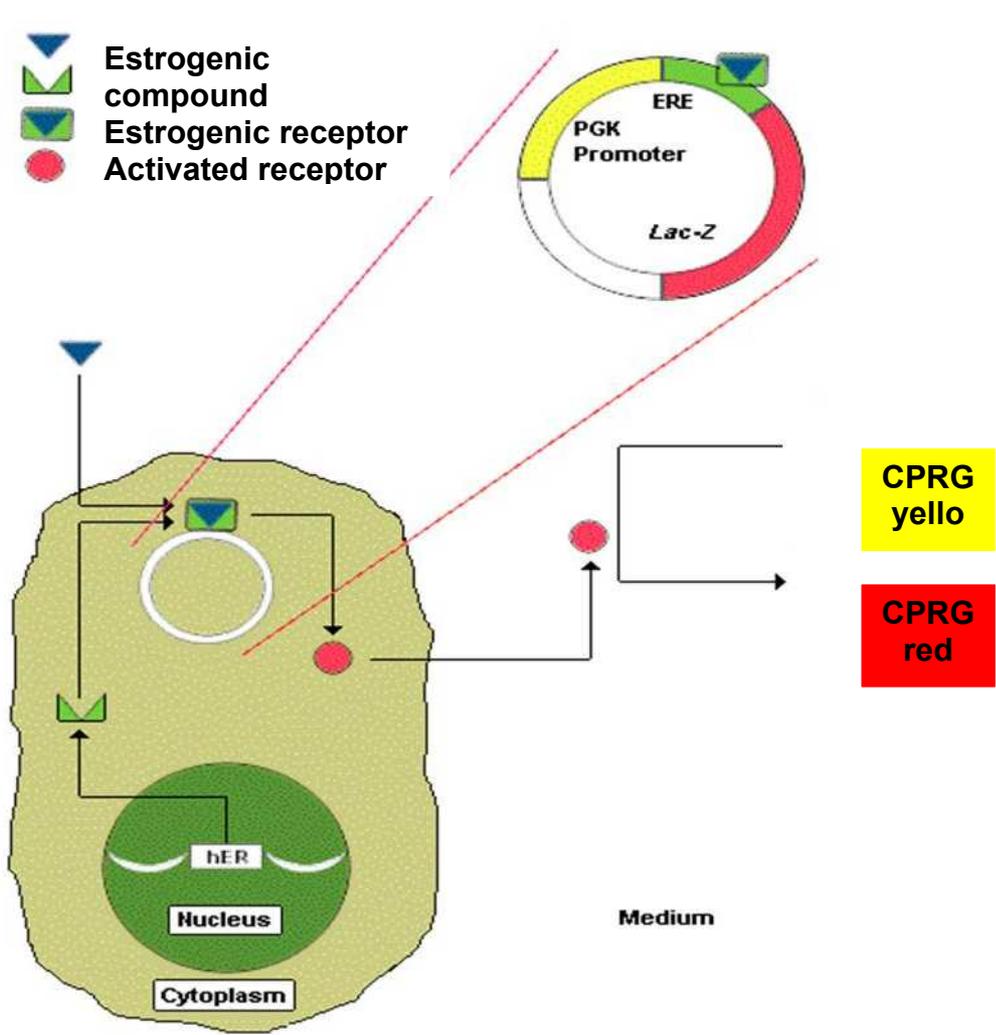


Fig. 3.7: Schematic Diagram of the Estrogen-Inducible Expression System in Yeast (After Routledge and Sumpter[91])

4. Materials and Methods

4.1. Quality control and validation of EROD assay

4.1.1. Intern laboratory quality control with Fly ash

For intern quality insurance, a sample of fly ash (FAMS) was at 1997 received from a municipal solid waste incineration station, and routinely analyzed in order to validate the intern tools and analytical instrument.

4.1.2. Interlaboratory comparison of dioxin-like compounds in food

4.1.2.1. General information

This intercalibration study is one of the first international exercises to validate the use of bioassays to determine the levels of dioxin-like compounds in food. It has been organized by Prof. Magnus Engwall group (Department of natural sciences, MTM centre Örebro University) in Sweden, and open for academic, regulatory and commercial laboratories.

We have participated to the first and second rounds of this interlaboratory comparison. The first round was performed during March to October 2002, 14 international laboratories (from Denmark, Germany, The Netherlands, Belgium, France, The Czech Republic, USA, and Japan) have participated, and the results were presented at the Dioxin 2002 meeting in Barcelona, Spain in August.

The second round intercalibration study, was a follow-up of the successful first one and took place during December 2003 to April 2004, 27 laboratories have registered, countries represented are Sweden, Norway, Denmark, Germany, The Netherlands, Belgium, Italy, UK, USA, Canada, Japan, Taiwan and New Zealand. Finally, the results were presented at the Dioxin meeting 2004 in Berlin, Germany, during November 2004.

4.1.2.2. Description of the bioassay

Bioassays were defined in this study as either a cell-based *in vitro* assay or kits, such as immunoassays, below is a short description of the bioassays used by the participating laboratories [132]:

DR-CALUX

This is a reporter gene-based bioassay using the luciferase gene under control of DER sequences. It is based on the H4IIE GUDLuc rat hepatoma cell line, which has been stably transfected with the plasmid pGudLuc1.1. The luciferase induction is measured after culturing and is correlated to TEQ exposure. The culturing time varies between 22 and 24 hours.

H4IIE-luc

This is a recombinant cell line containing a luciferase reporter gene under control of DER sequences. It is based on the H4IIE-luc rat hepatoma cell line, which has been stably transfected with the plasmid pGudLuc1.1. The luciferase induction is measured after culturing and is correlated to TEQ exposure. The culturing time is 72 hours.

CALUX and DIPS-CALUX

This is also a reporter gene-based bioassay using the luciferase gene under control of DER sequences. It is based on the Hepa 1 mouse hepatoma cell line, which has been stably transfected with a plasmid containing the luciferase gene under control of DER sequences.

The abbreviation DIPS stands for dioxin/furan and PCB specific, which according to the participating laboratories is a selective clean-up method to isolate PCDD/F from PCB. After *in vitro* cell culturing, luciferase induction is correlated to TEQ exposure. The culturing time is 20-24 hours.

RTL-W1

This is a cell-based bioassay using induction of EROD activity. It is based on the cell line RTL-W1 (rainbow trout liver- Waterloo1). It is based on the principal that after *in vitro* cell culturing. EROD induction is correlated to TEQ exposure.

MH1C1 EROD assay

This is a cell-based bioassay using induction of EROD activity. It is based on the cell line MH1C1, which was originally isolated from a rat hepatoma. After cell culturing, EROD induction is correlated to TEQ exposure. The culturing time is 24 hours.

Immunoassay

PCDD/F are specifically bound by anti-dioxin antibodies, which are immobilized on the EIA tube surface. Unbound material is washed away, and a competitor enzyme is added which binds to the free sites of the antibodies. The amount of conjugate is inversely related to the amount of PCDD/F bound on the EIA tube.

4.2. Application of micro-EROD bioassay

4.2.1. Samples collection information

4.2.1.1. Samples for Interlaboratory study

The following samples were tested:

In the first round 2002:

- 15 g Cod liver (fished in Skagerrak)
- 10 g Fly ash sample from a municipal solid waste incinerator
- Extract from 1 g fly ash, dissolved in 1 mL toluene

In the second round 2004:

- 30-35 g of homogenized salmon muscle,
- 140 µL of PCDD/F + non-ortho PCB mixture,
- 140 µL of PCDD/F + non-ortho PCB + mono-ortho PCB mixture.

Before shipment to the participants, the biological homogenates was thawed, homogenized once more and were placed, in form of aliquot; in scintillation glass vials. The fly ash sample was placed in an amber glass vials (10 mL). The fly ash extracts and (PCDD/F + non-ortho PCB + mono-ortho PCB mix) were distributed in a small glass ampoule.

The frozen samples for each laboratory were placed in metal container, which also was filled with adsorbent material in case of leakage.

4.2.1.2. Human milk samples

The human milk samples are a part of the research project EXPORED, which concerns exposure-outcome relationships in male urogenital malformation with special references to EDCs. The earlier cohort studies in Denmark and Finland showed the two countries with different incidences of male urogenital disorders, such as cryptorchidism and hypospadias [133], the boys with malformations had been diagnosed at birth and followed up to 18 months together with matched controls. Biological samples had been systematically collected during these studies for exposure assessment. Comprehensive endocrine evaluation of the children had also been performed, and a large database had been created on the basis of questionnaires. The aim is to combine these databases with the exposure data of endocrine disruptors to assess their roles in the formation of male urogenital malformations. The mother exposure data to PBTs, such as PCDD/F, PBDE, PCB, phthalates, alkylphenols, Bisphenol A, halogenated hydrocarbons and selected pesticides, will be used to make a risk analysis combining the other database.

The study was approved by the local ethics committee and conducted according to the Helsinki II declaration.

The project organizer collected the milk samples. The protocol of the samples (Finnish milk 4-6 weeks and Danish milk 4-12 weeks post partum) preparation was carried as following:

1. Open the cork/cap/stopper of the frozen bottle
2. Place the bottle in a “minigrip” pack or such like (other glass) in order to avoid possible loss of sample if bottle will break during defrosting
3. Defrost the bottle in a fridge
4. Temperate the bottle to room temperature after thawing
5. Shake/mix the sample at 30-40°C for 1 hour
6. Close the cork/cap/stopper of the bottle and shake vigorously
7. Aliquot the sample
8. Freeze the aliquots as soon possible and send them to other laboratories on dry ice.

2 x 20 mL milk sub-samples, sealed in scintillation glass with aluminum padded cap, was accepted by IÖC (Institute for Ecological Chemistry), GSF for analysis. When starting analysis, a pooled control sample of milk (1-2 L) has been provided by Copenhagen to establish the sample preparation procedure.

The first 20 mL of samples were destined to chemical analysis of halogenated Hydrocarbons and chiral persistent by Mr. He-Qing Shen [134], the second 20 mL were analyzed for dioxins and dioxin-like determination by EROD-Bioassay, and the chemical analysis of dioxins and PCB was performed by other project partners.

4.2.1.3. Leachate-polluted groundwater sample

These samples are a part of pilot project for investigation of pollution evaluation of groundwater, surrounding final disposal site Le Letten and Roemislochb in Elsass/France. The samples collection was performed by engineer's office (ANTEA) on behalf of IG DRB (Interessengemeinschaft Deponiesicherheit Regio Basel), from 3rd to 5th November 2004, and shipped to the project partners for different toxicity and biodegradation analyze.

6 x 5 L glass bottles (schott) filled by collected samples and one empty bottle, were accepted by IÖC (Institute for Ecological Chemistry) GSF for dioxin and estrogenic activity analysis.

4.2.1.4. Q1 and TBA compounds

Q1 (2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole) was synthesized as recently reported [12,18] by Prof. Vetter in Institute of Food Chemistry University of Hohenheim, and (TBA 2,4,6-tribromoanisole) was ordered from Aldrich (Taufkirchen, Germany). 10 mg of each compound as a solid were shipped to IÖC for investigation.

4.2.2. Samples preparation

The samples preparation takes place in dioxin laboratory, GSF-Research Centre, Institute for Ecological Chemistry, (Neuherberg, Germany). Dioxin laboratory is an

accredited laboratory against the standard DIN EN ISO/IEC 17025 [135]. That certificate provides for the dioxin laboratory a guarantee of the quality of its measurements. It demonstrates the competence of this testing laboratory to carry out specific tests.

The whole samples preparation procedure explained below are carried out according to the corresponding SOP (standard operated procedure). These SOPs are verified against the above-mentioned standard and all rights are reserved from dioxin laboratory.

Once registered in dioxin laboratory sample is processed for further extraction and clean-up steps.

4.2.2.1. *Lipid determination for biological samples*

Evaporate the solvent by rotary vacuum evaporator with a water-bath temperature up to 45 °C and a mediate rotary rate, the vacuum was controlled around 600 mbar. When the extract was condensed to about 0.5 mL, removed the round bottom flask from the evaporator, and then evaporated it to hemi-dry with mediate stream of nitrogen. After that, the flask was placed for 6 hours into desiccator until stable weight was achieved (i.e. error of two separate weight measurement over at least two hours $\pm 0.0005\text{g}$). The lipid content was calculated on the base of wet weight sample.

4.2.2.2. *Extraction*

Samples for Interlaboratory study

Fly ash samples contain a high carbonate amount, therefore before extraction; 0.5 g of sample was first treated with 20 mL of 20% HCl until the end of CO₂ gas formation. Then the sample was placed in an Ultrasonification bath for 1 hour, finally the suspension was filtered, and washed many times until the pH of the filtrate is between 6 and 7. The remains were placed in a desiccator and drying over the night.

For COD liver and Salmon, the samples first were semi-dried and homogenized by mixing with Na₂SO₄, and then placed in a cellulose extraction cells.

The extraction step of all the samples was performed following the ASE method using hexan/acetone 57:25 (v/v) (2X10 min, p=120 bar).

Human milk sample

10 g of mother milk sample were semi-dried by mixing with Sodium sulfate/sea sand mixture in mortar.

The homogenized mixture was transferred to a column, the mortar, spoon and pestle were carefully washed using some sulfate/sea sand mixture, which should also be transferred to the column. The packed materials were eluted with 250 mL (acetone/hexane 2:1 v/v) mixture. The obtained extract was concentrated by evaporation, and transferred to a column for a clean-up step.

Groundwater sample

100 mL of Toluene were added to 5 L of Water samples and mixed for 48h. After phase separation, the water phase was removed. Two extracts of toluene were combined to one sample and dried over Na₂SO₄. Finally, the solvent volume was reduced by evaporation, and the extract was subdivided in two parts. The first one was tested directly in EROD assay. The second part of concentrated extracts was applied to a column for a further clean-up step.

For estrogen activity screening, was followed the same procedure as described in the extraction for the EROD bioassay, three picograde extraction solvents were used: benzene, hexane and dichloromethane. Water sample was also directly tested without further treatment.

For Quality control of the clean-up and extraction procedure a commercial mineral water samples (Fachinger) were treated in parallel, in addition glassware blanks were prepared.

4.2.2.3. *Clean-up step*

Sandwich column

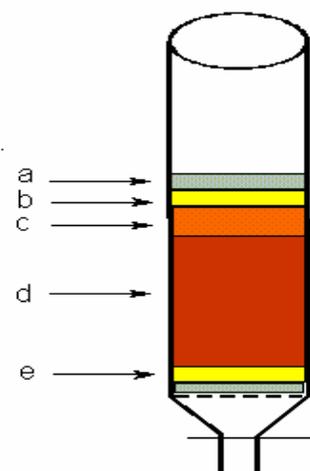
On the sandwich column, many organic compounds are oxidized by reaction with the sulfuric acid. The resulting polar bonds are absorbed at the silica gel and are eluted

with the non-polar solvent n-hexane. This clean-up step oxidizes poly-aromatic hydrocarbons (PAH).

Preparation of the sandwich column:

The column will be filled from top to bottom with the following materials:

- a) 10 g water-free sodium sulfate.
- b) 10 g active silica gel (mesh 63–200 μm).
- c) 20 g silica gel (44% concentrated sulfuric acid w/w).
- d) 40 g deactivated silica gel (4% water w/w).
- e) 10 g water-free sodium sulfate.



Elution of the sample:

In order to avoid blank values the packed sandwich column is rinsed with 60 mL n-hexane. Before the upper layer becomes dry, the sample is transferred with a Pasteur pipette to the packed column.

Finally, the sample volume is reduced at the rotary evaporator in a bath with temperature 60°C and at a pressure from 500 to 550 mbar. The final sample (approx. 2 mL) should be colorless or less colored than the initial fraction.

Samples were eluted with 870 mL n-hexane and 8.7 mL dichloromethane, the eluate reduced by evaporation (550 mbar, 333 K) to 2–3 mL. The extract was transferred stepwise into a vial with 200 μL DMSO and evaporated under a stream of nitrogen. Finally after evaporation, 200 μL DMSO and 200 μL Isopropanol were added, the result is that the sample is dissolved in 500 μL DMSO/isopropanol (4:1 v/v).

4.2.3. Bioassay description: Micro EROD

The micro EROD test is executed in the ecotoxicology laboratory (S1 security level) in Institute for Ecological Chemistry, (Neuherberg, Germany), the cell culture and the

bioassays were performed under sterile conditions in a laminar flow bank and carried out according to the corresponding standard operated procedure (SOP_Z01-/07)

4.2.3.1. Cell culture

The H4-IIIEC/T3 rat hepatoma cells were derived from the Reuber hepatoma H-35, and at 1977 supplied from Dr. Thompson (NCL, Bethesda, USA) to Institute of Toxicology in GSF- research center.

The cells were cultured in a Dulbecco's MEM medium (DMEM), containing 1.0 g/L α -D-glucose, 3.7 g/L NaHCO_3 , and 1.0289 g/L N-acetyl-L-alanyl-L-glutamine, supplemented with fetal bovine serum, penicillin, and streptomycin. The cell culture and EROD assay were based on Donato's method. Cells were seeded into individual wells of a 96-well microtiter tissue culture plate. Cells were grown for 24 h to about 60–70% confluence, and then exposed with the sample for 24 or 72 h. The plate was incubated at 37 °C and 7.5% CO_2 . For comparisons, cultures were treated with various concentrations (0.4 pM – 12.4 pM) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD).

4.2.3.2. Ethoxyresorufin-O-dealkylase assay (EROD)

EROD enzyme activity was determined directly in intact rat hepatoma cell cultured on 96-well plates. The old medium with samples was removed and the assay was started by the addition of 100 μL /well of fresh culture medium containing 8 μM of 7-ethoxyresorufin as substrate and 10 μM dicumarol. During the incubation 7-ethoxyresorufin will be deethylated by the induced CYP1A1 and converted to a fluorescent Resorufin. The fluorescence follows a sigmoid concentration response relationship with 2,3,7,8-TCDD concentration.

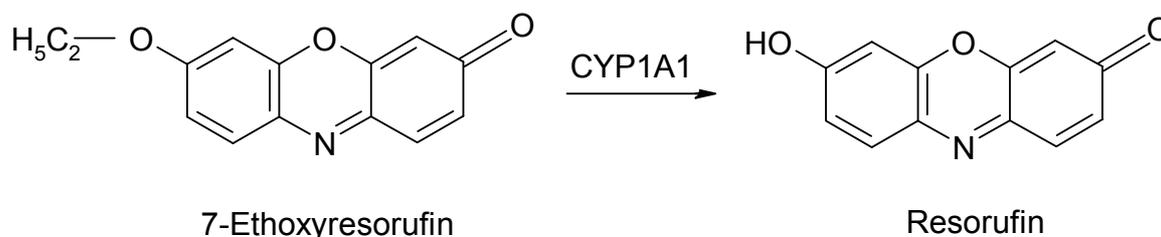


Fig. 4.1: CYP1A1-Deethylation reaction of 7-Ethoxyresorufin

After 30 min incubation at 37 °C without cover in sterile incubator, a 100 µL aliquot of cell medium was withdrawn from each well and transferred to another 96-well plate containing 200 µL of ethanol/well. Fluorescence of the product resorufin was recorded directly on fluorescence microplate at 550 nm excitation and 585 nm emission wavelengths, which could be regarded as relative EROD induction value. After Fluorescence measurement, the cell cultures were washed with PBS and stored in –20 °C for 24 h. Then, the plate were defrosted by adding 150 µL PBS and used for determining the cytotoxicity of the test materials employing the Resazurin test and for protein test using BCA-method.

4.2.3.3. Protein amount determination: BCA-Method

Protein amounts were assayed according to Smith et al 1985 and performed in micro plate with BCA-standard curve. 100 µL of protein sample was mixed with copper sulfate solution (50:1 v/v) (green solution) and incubated for 45 min at 37 °C. Under alkaline reaction conditions and in presence of proteins, Cu (II) will be reduced to Cu (I). Cu (I)-Bicinchonin acid complex (purple color) will be formed and its adsorption was measured at 540 nm.

4.2.3.4. Cytotoxicity determination

Principal

The cytotoxicity test, also called resazurin-test, is performed for detection or evaluation of cytotoxicological effects, and carried out after the EROD bioassay. It is based on the metabolism capacity of the intact cells to reduce the blue resazurin indicator to red resorufin. The fluorescence of the red resorufin correlated directly, with the number of the still living cells with 85 % of the intact cells the test still to be exploited [136].

Experimental

After the EROD-Bioassay the cells were, with 50 µL /well of 1.2% Glutataldehyde solution, fixed at the bottom of the micro plate. After 5-10 min, this solution was removed by two times washing with 200 µL /well of PBS. Finally, 200 µL /well resazurin solution/DMEM culture media per well was added by multi channel pipette. After 90 min incubation at 37 °C with cover, the fluorescence measure is performed

at 550 nm excitation and 585 nm emission wavelengths. The Fluorescence units were in percent to a control sample (DMSO/Isopropanol 4:1 v/v) presented.

Finally, the plates containing the cells were washed 2 times with 200 μ L PBS and stored at -20°C .

4.2.3.5. Data Calculations

The EROD bioassay consists of two test systems: Measure of Specific EROD activity and protein amount determination according to BCA method. The obtained measurement data will be transferred and copied in an Excel calculation file. From the quadruplicates of the fluorescence and absorption measurements data, for each concentration the mean of triplicates will be calculated. From this mean value, the blank value will be subtracted. The corrected absorption value will be calculated after the linear regression of protein concentrations ($\mu\text{g/mL}$).

Finally, the specific EROD activity is calculated using the following formula:

$$\text{Specific EROD activity} = (\text{Fluorescence corrected} / (a * \text{mg Protein} * \text{Incubation time}))$$

Definition of terms: *a* = specific apparatus-value (21.35, TECAN-Spectrafluor)
Incubation time = Incubation of 7-Ethoxyresorufin with induced cells (30 min)

EROD activity is expressed as picomoles of resorufin formed/mg. min

The EROD activity of 2,3,7,8-TCDD standards is plotted against the log (concentrations) and presented as a sigmoid curve (Fig. 4.2).

Dose response curves for EROD activity is computed by non-linear regression using the classical logistic sigmoid curve as model equation [137].

4-parameter equation:

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D$$

Definition of Terms: *A* = *Y* value of lower asymptote

$B = \text{relative slope of middle region}$
 $C = X \text{ value at midpoint of curve}$
 $D = Y \text{ value of upper asymptote}$

Finally, the biological TEQ is determined by comparing the induction of EROD activity caused by environmental samples with that caused by authentic TCDD standards (0–0.4 pg TCDD/well) [138] The TEQ values determined in duplicate experiments.

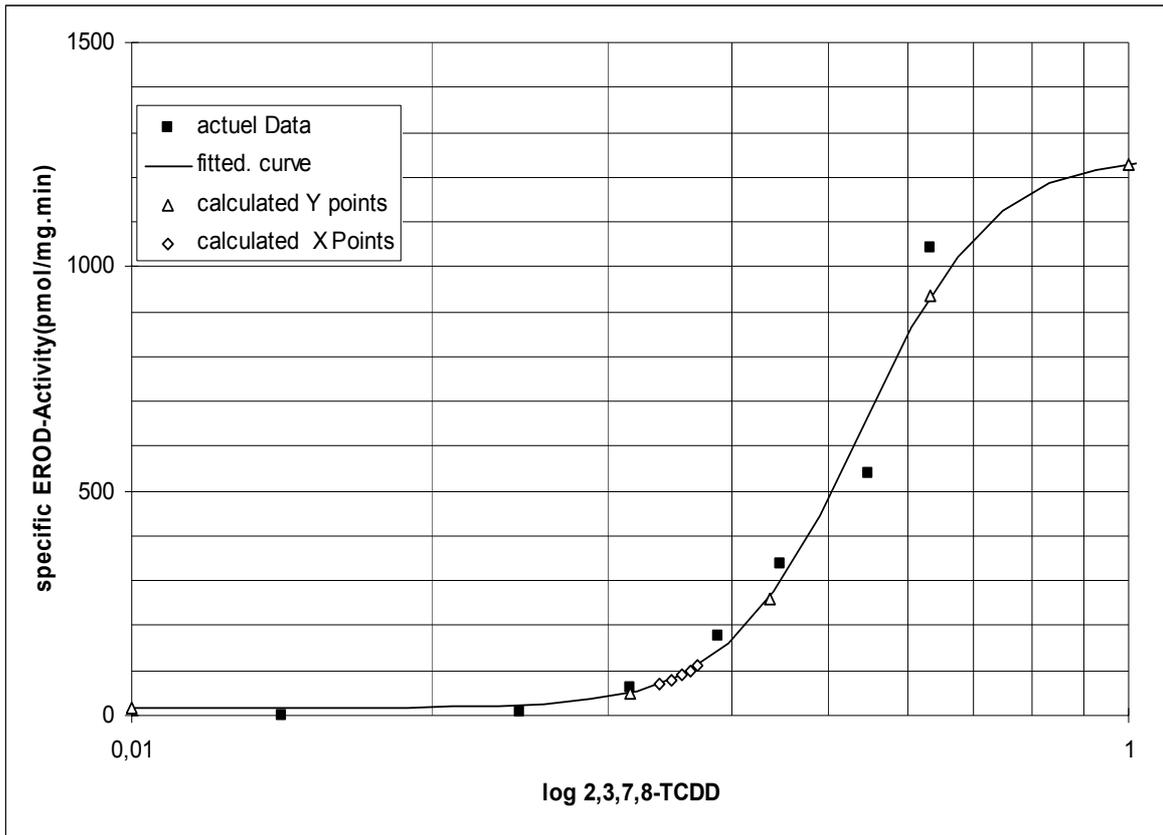


Fig. 4.2: 2,3,7,8-TCDD standard curve in micro-EROD bioassay

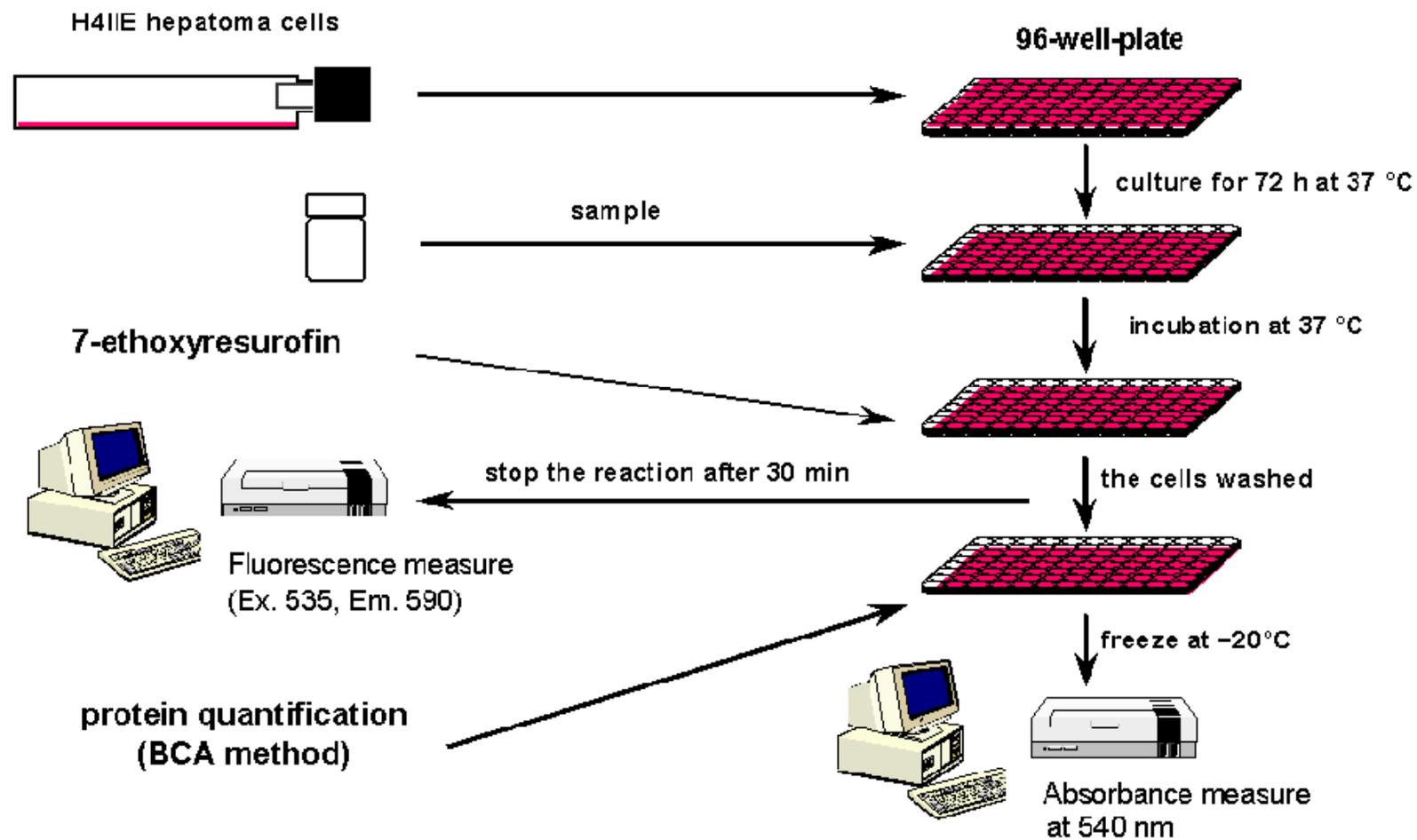


Fig. 4.3: Experimental procedure of EROD-Assays

4.3. Development of a bioactivation protocol

4.3.1. Modification of recombinant yeast assay

4.3.1.1. Recombinant yeast assay

The recombinant yeast assay takes place in the ecotoxicology laboratory (S1 security level) in Institute for Ecological Chemistry, (Neuherberg, Germany) separately to the EROD micro assay laboratory in order to avoid any cells contamination risk. The cell culture and the bioassays were performed under sterile conditions in a laminar flow bank and carried out according to the corresponding standard operated procedure (SOP_M03)

4.3.1.2. Yeast stock production

Saccharomyces cerevisiae strain was kindly provided by Professor J. Sumpter (Brunel University, UK) to produce the 10x yeast stock, four 50 mL cultures were grown (growth medium: see annex) to an optical density of 1.0 at 640 nm and transferred to a sterile 50 mL centrifuge tubes. The cultures were centrifuged at 4°C for 10 min at approximately 2000xg. The supernatant was decanted and the separate cell batches were resuspended in 5 mL fresh minimal medium with 15% glycerol. The yeast strain was stored at -80°C (Long time storage maximal 2 years) or -20 °C (short time storage: maximal 4 months) in 0.5 mL aliquots in 2 mL sterile cryogenic ampoules. Long storage Growth curve of *Saccharomyces cerevisiae* strain is shown in Fig. 4.4.

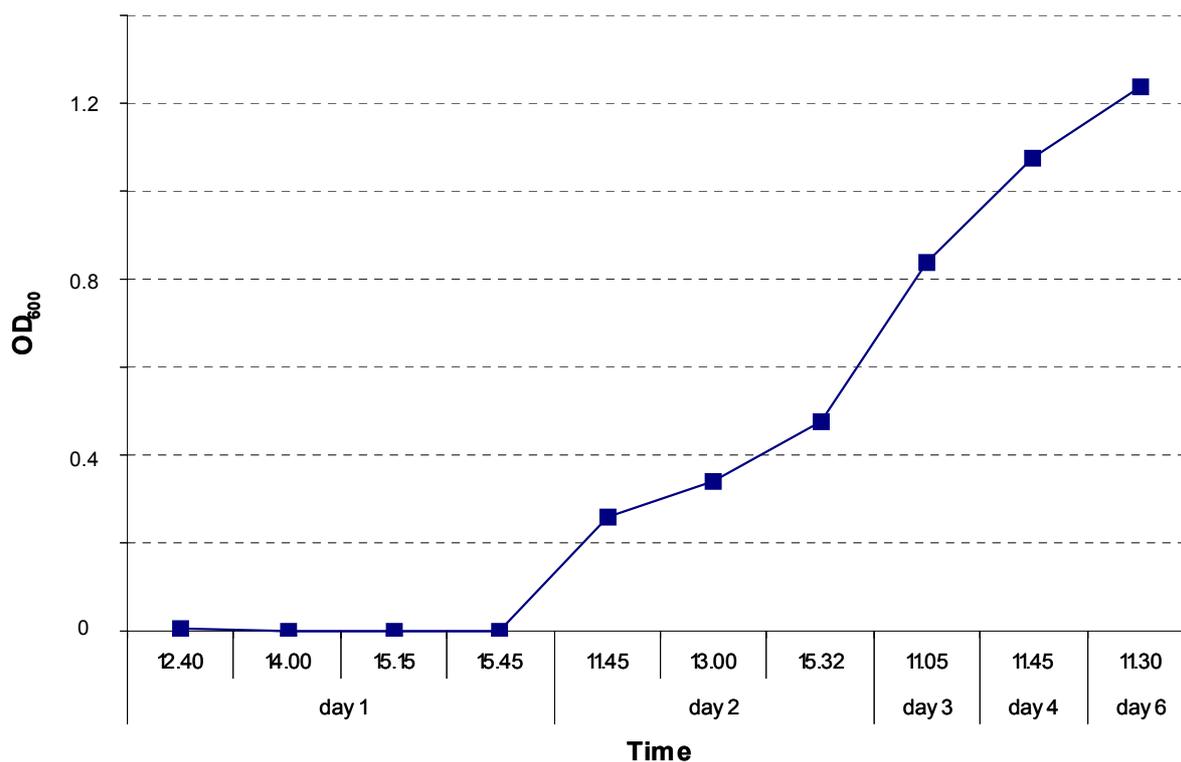


Fig. 4.4: Growth curve of *Saccharomyces cerevisiae* (Sumpter-strain) [139]

4.3.1.3. Standard assay procedure:

Growth medium was prepared by adding 5 mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-threonine solution and 125 µL copper (II) sulfate solution to 45 mL minimal medium in a sterile conical flask. At day zero, 50 mL of growth medium was inoculated with 125 µL of the 10x yeast stock and incubated at 28°C for 24 hours on an orbital shaker (150 rpm) or until an absorbance of 1.0 at 620 nm was reached. The following day, assay medium was prepared by adding 0.5 – 2 mL of the 24 yeast culture and 0.5 mL of a CPRG solution (10 mg/mL) to 50 mL of growth medium (yeast concentration approximately 4×10^7 cells/mL). Stock solutions of chemicals were serially diluted in absolute ethanol, and 10 µL aliquots of each concentration were then transferred in duplicate to separate 96-well optically flat bottom microtiter plates. The ethanol was allowed to evaporate to dryness on the assay plate, and 200 µL of assay medium were added. Each plate contained one standard curve dilution of 17-β-Estradiol and solvent control.

The plates were incubated in a naturally ventilated heating cabinet at 32°C. After three days of incubation, all wells were homogenized and further incubated for the appropriate time. The enzymatic reaction was followed at an absorbance of 540 nm. Yeast growth was checked using a second reading at 620 nm.

Data Calculations

The values are determined in quadruplicates, mean of arrangement of triplicates. Dose response curves for β -galactosidase activity were obtained using corrected Absorbance:

$$\text{Corrected Absorbance} = (\text{Abs540}) \text{ compound} - [(\text{Abs620}) \text{ compound} - (\text{Abs620}) \text{ blank}].$$

The best fitting curve was calculated using Excel 2003 program, by a four parameter logistic regression as advocated by Scheinost [137], according to the formula above used for EROD activity determination.

4.3.1.4. Modified Assay procedures of recombinant yeast assay

The standard assay procedure was modified in a number of experiments series, in order to be compatible and good combined with the metabolic activation protocol. Therefore the following modifications were performed:

- the effects of reduced incubation period
- the solvent change
- mode of addition of test chemical on the assay response

In all the experiments, 17 β -estradiol has been used as reference for protocol optimization.

Effect of incubation time

Some plates were incubated for longer than 3 days. These plates were incubated at 32°C for the first 3 days; the enzyme activity was measured every day during the incubation time

Effect of solvent and addition mode of the test chemical

The solvent of the test chemical was changed from Ethanol to DMSO/isopropanol (4:1 v/v) and the estrogenic activity was compared.

In the standard assay procedure, the vehicle (ethanol) was allowed to completely evaporate (leaving the test chemical dried in the well) prior to the addition of the medium. When in the modified protocol, the 17 β -Estradiol was dissolved in solvent and added directly to the medium. Five μ L of test chemical dissolved in DMSO/Isopropanol (4:1 v/v) and 195 mL of medium (containing yeast and CPRG) were used, giving a final concentration of 2.5% of the solvent.

4.3.2. Bioactivation protocol

The development of the bioactivation assay, is performed by steps, the first proposed experiment protocol was the following (Fig. 4.5):

Day 1:

- Cells in large cultivation bottles trypsinised.
- Cells counted and number of cells per mL determined.
- Cells seeded into 25 cm² cultivation bottles, 500 000 cells/bottle.
- Medium used: 5 mL of Dulbecco's modified minimum essential medium. (DMEM) without phenol red, supplemented with 10% of fetal calf serum (FCS).

Day 2:

- 25 μ L of 2,3,7,8-TCDD solution.

Day 3:

- Medium exchanged for DMEM without phenol red supplemented with 10% of charcoal stripped FCS (= hormone free FCS); 5 mL of medium used.
- 25 μ L of tested compound solution (mostly BPA 1mg/mL) added to each bottle for metabolization; final concentration = stock solution concentration/200.

Day 4:

After 24 hours of incubation cells were frozen.

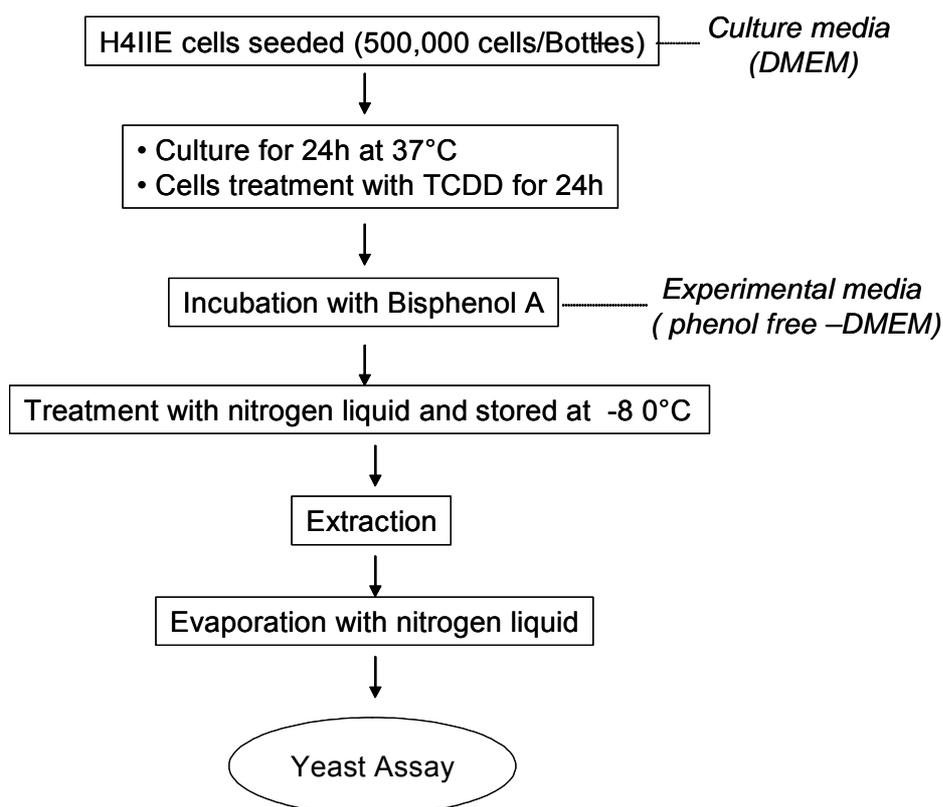


Fig. 4.5: Experimental protocol of the proposed bio-activation System

4.3.3. Optimization of the metabolites extraction

The procedure of metabolites extraction is carried out in many steps (Fig. 4.6) and described as following:

- After metabolic activation step, the H4IIE cells and culture media, containing Bisphenol A and its metabolites, were frozen and thawed again, cell wall further destroyed and enzyme activity eliminated (protein denaturation) by 10 minutes of sonication using ultrasonic bath.
- Estimation of extraction recovery: to bottles without metabolized BPA added (after freezing, thawing and sonification) 25 μ L of the same solution as for metabolic de/activation and left to equilibrate for 1 hour, blank experiment is carried out in parallel without any addition of BPA even after the sonication.

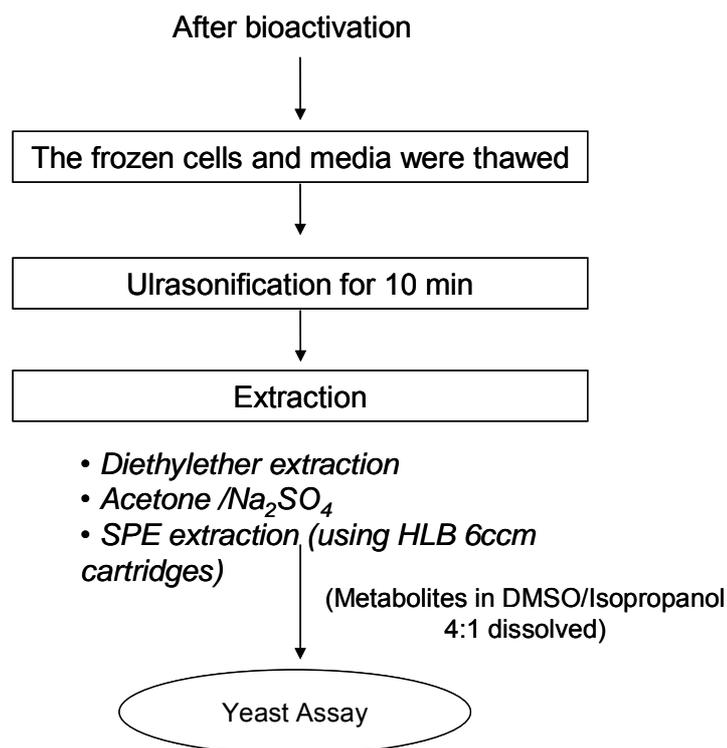


Fig. 4.6: Experimental Protocol of extraction procedure of metabolites

The metabolites extraction is optimized by testing three different extractions methods, described as following:

Diethyl ether extraction:

- bottle content was transferred into 12 mL centrifugation tube
- bottle was rinsed respectively with two solvents: First 2 times with 1mL of distilled water, then 2 times with 1.5 mL of diethyl ether (DEE)
- the rinsed solvent were collected and added to the contain of centrifugation tube
- tubes were vigorously shaken for two minutes
- centrifuged for 5 minutes by 900 rpm
- the upper layer was transferred into 20 mL glass vial:
 - in DEE layer could be seen rest of proteins creating a gel-like layer
 - always taken away as much of DEE as possible, then 2 times rinsed with 2 mL of DEE
- the supernatant was extracted 2 times more with 3 mL of DEE, always rinsed with additional ether

- DEE evaporated under nitrogen stream to ca. 200 μ L, quantitatively (rinsed about 5 times by 200 μ L of DEE) and transferred into a 1.5 mL glass vial
- the final volume was evaporated to minimum volume, afterwards 200 μ L of DMSO was added
- then, the obtained sample was evaporated to final volume 200 μ L, then 50 μ L of isopropanol added (final volume 250 μ L, 10x diluted in comparison with original solution added to cells)

Acetone/ Na_2SO_4 extraction:

- to the bottle with medium and cells added 15 mL of mixture acetone: DEE (4:1 v/v)
- 15 g of dried Na_2SO_4 added directly to the bottle
- placed in ultrasonic bath for 5 minutes
- the liquid phase transferred into 20 mL glass vial
- extracted twice more with 5 mL of acetone:DEE
- under stream of nitrogen evaporated; rest of water removed by further addition of 4 g of Na_2SO_4
- organic phase transferred into another 20 mL vial, the first one rinsed 3x by 5 mL of acetone: DEE
- evaporated to minimum volume, transferred into a 1.5 mL glass vial, rinsed 5x with 200 μ L of acetone
- evaporated to minimum volume, afterwards 200 μ L of DMSO was added
- evaporated to final volume 200 μ L, then 50 μ L of isopropanol added (final volume 250 μ L, 10x diluted in comparison with original solution added to cells)

SPE extraction:

- HLB 6ccm cartridges used
- conditioned with 6 mL of methanol
- equilibrated with 6 mL of water
- sample applied, bottle rinsed 2x by 1mL of distilled water
- glass vial for waste exchanged for 20 mL glass vial for elution
- bottle rinsed 2x with 1 mL of methanol and applied on the cartridge
- elution using next 8 mL of methanol

- evaporated to reduce the solvent volume, transferred into a 1.5 mL glass vial, rinsed 5x with 200 µL of methanol
- evaporated to minimum volume, afterwards 200 µL of DMSO added
- evaporated to final volume 200 µL, then 50 µL of isopropanol added (final volume 250 µL, 10x diluted in comparison with original solution added to cells)

Extraction recovery calculation of BPA measured using YES assay

The extraction yield is measured using no metabolized BPA by Yeast Assay, and calculated as following:

$$\text{Extraction yield (\%)} = \frac{(\beta\text{-Galctosidase Activity of extracted BPA} - \beta\text{-Galctosidase Activity of the blank})}{(\beta\text{-Galctosidase Activity of not extracted BPA} - \beta\text{-Galctosidase Activity of the blank})} \times 100$$

4.3.4. Metabolic activation assessment without extraction

In order to avoid the risk of metabolites lost and solvent consummation during the extraction, the bioactivation protocol was modified by avoiding this step, the modified protocol is the following:

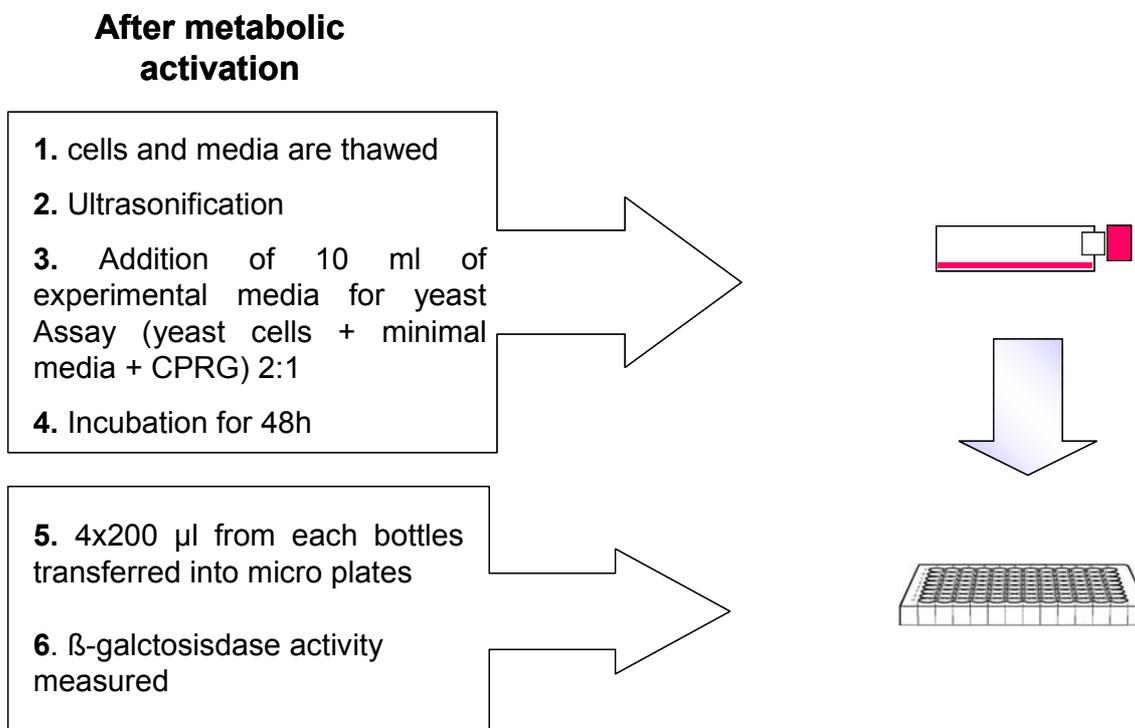


Fig. 4.7: Modified experimental protocol without extraction step

4.3.5. Automatisation of the Bioactivation assay (96-Well-plates)

The experimental protocol of the micro-bioactivation assay is reported in the Fig. 4.8, this automatisation of the system on 96 well plates, allows the performance of two analyzes in parallel, that mean the estrogenic activity of the system, by yeast assay and the Induction of CYP1A enzyme activity by micro-EROD assay.

4.3.6. β -Glucuronidase Assay

Samples were subjected to enzymatic hydrolysis to identify possible glucuronide conjugates of Bisphenol A. Samples were incubated at 37°C for at least 24 h with – glucuronidase (2000 U/mL) according to the method of Peters and Caldwell [140].

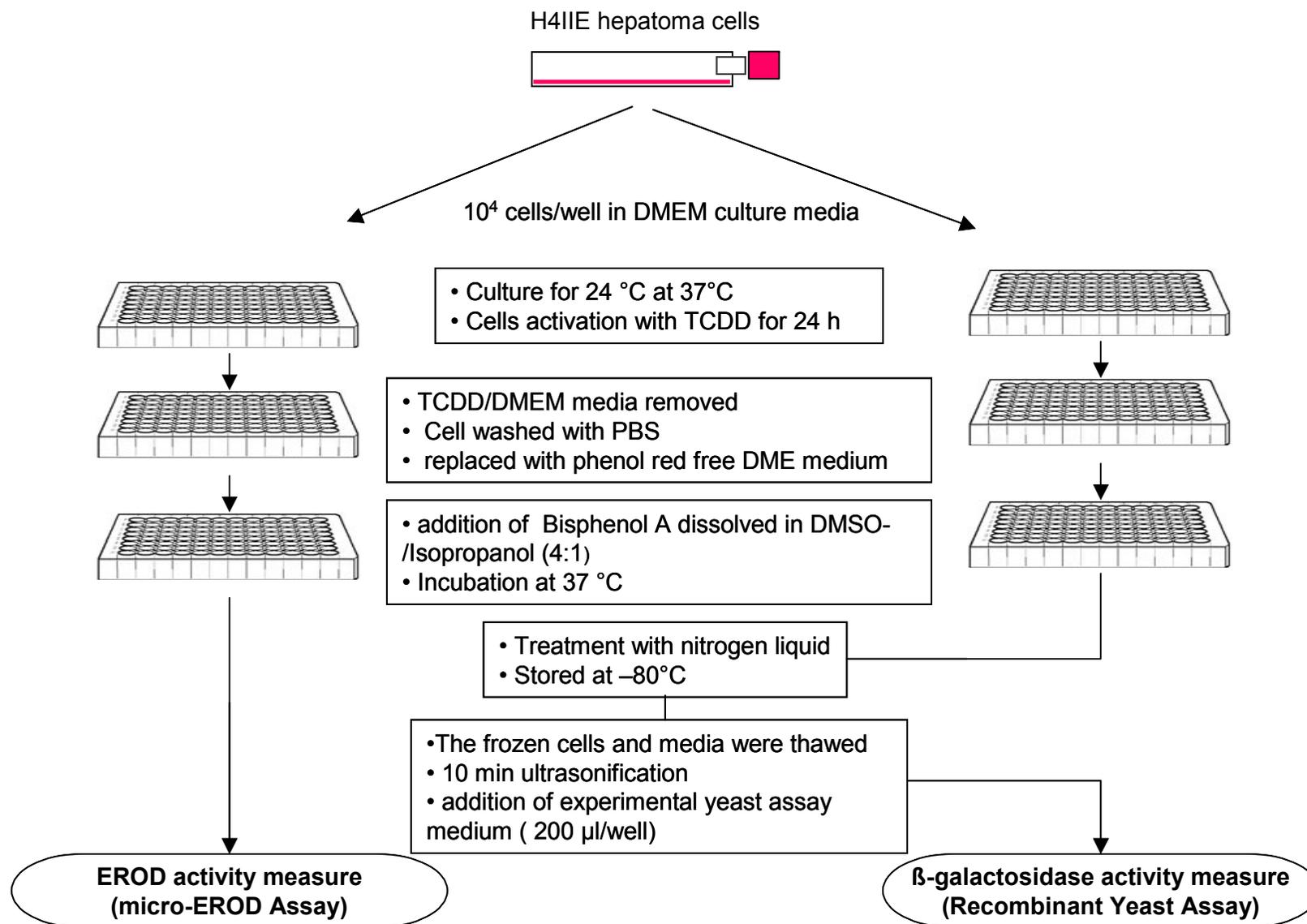


Fig. 4.8: Experimental procedure of bioactivation system performed on 96 well plates

5. Results and discussion

5.1. Quality of data of micro-EROD bioassay

The micro-EROD bioassay is performed in 96 well-plates, each plate contain Blanks and samples: quadruplicates; mean of "best" triplicates, the mean of blanks are automatically subtracted from the mean of samples.

For intern quality assurance, a fly ash sample (FAMS) was routinely analyzed in order to validate the intern tools and analytical instrument (data not shown)

EROD-bioassay statistics for effect estimates are reported as following:

EC50 for TCDD	pM	pg (total amount required for an EC50 determination)	
	6.2	0.945/curve	
Minimal detection limit for TCDD (pM)	pM	pg (total amount required for detection)	
	3.1	0.1/well	
Throughput capacity (number of samples/week)	Extraction and clean-up	Bioassay (complete curves)	Bioassay (screening)
	6-10	10	120
EC50 determination for TCDD (within 9 different micro plate assays)	Standard deviation for one determination	95 % confidence interval for one determination	
	2%	16%	
Assay to assay variation (%)	EC50 determinations for TCDD	TEQ determinations in complex environmental samples	
	maximum: 10-15	maximum: 15	

5.2. Validation of micro-EROD bioassay in intercalibration study

The EROD bioassay has been developed and used since many years in laboratory of Institute of Ecological Chemistry in GSF- research centre, the experimental protocol was also optimized during this period, for example sample preparation, clean up procedure, macro- EROD test automatisation on 96 well plates (micro-EROD) [48, 136,141, 142] and an intern validation was always performed.

The participation to the first and second rounds of interlaboratory comparison of dioxin-like compounds in Food using bioassays was the first exercise for an international validation of our bioassay in comparison with other test.

In intercalibration study, each participant has a code. Our laboratory has a code 7 during the first round and 12 during the second one. Furthermore our laboratory was the single participant that used micro EROD bioassay. All the results of the other participated bioassays, reported in this part of thesis, were supplied by Dr. Engwall.

5.2.1. Bioassays sensitivity

The laboratories also reported various measures of bioassay performance, including EC_{50} value for TCDD and amount of TCDD required for detection (Table 5.1). Thus, the most TCDD-sensitive bioassay was the RTL-W1, followed by micro-EROD bioassay. The EC_{50} value is 6.2 pM and the amounts of TCDD required for detection is 0.10 pg (Table 5.1).

Table 5.1: EC50 and amounts of TCDD required for detection in the different bioassay in the first round of the study.

Lab Code	Bioassay	EC ₅₀ for TCDD (pM)	Amount required for detection (TCDD) (pg)
1	CALUX	-	-
3	DIPS-CALUX	43.20	0-100
15	CALUX	-	1-20
8	DIPS-CALUX	43.20	-
2	DR-CALUX	10	0.060
13	DR-CALUX	9.5	0.064
11	DR-CALUX	20	0.019
16	DR-CALUX	0-300	0.021
10	DR-CALUX	10	0.020
7	H4IIE-luc	27.60	0.120
5	RTL-W1	3.2	0.160
17	RTL-W1	4.2	-
4	MH1C1 EROD assay	11.9	20
12	Micro-EROD Bioassay	6.2	0.100

5.2.2. Bioassays performances

Six samples were analyzed by micro-EROD assay, the results were compared with the performance of the other laboratories, and those generated from chemical analysis.

During the first round of the study, the cod liver sample was analyzed by 12 laboratories of which four used the CALUX assay, five the DR-CALUX assay, two the RTL-W1 bioassay while the H4IIE-luc and MH1C1 EROD were used by one laboratory each. The concentration of TEQs determined by the laboratories ranged between 0.2 and 28.7 pg/g fresh weight (Fig. 5.2).

The concentration of PCDD/F, non-and mono-ortho PCB, expressed as WHO-TEFs were 27 pg/g wet weights, no consistent difference in TEQ levels between the different bioassay types could be seen. This is not surprising since all the bioassays used to perform the cod liver analysis, are based on rat and mouse hepatomas.

Overall, eight of the twelve laboratories had values that were between 60 and 106% of the WHO-TEQ value.

The TEQ measured by micro-EROD bioassay was 18.8 ± 2.9 pg/g fresh weight and present 70% of the WHO-TEQ value. Similar performance of micro-EROD was registered during the second round of the intercalibration study, by analyzing salmon sample (Fig. 5.1). The measured TEQ was 11.6 ± 0.7 pg/g fresh weight and present 77% of the WHO-TEQ value (TEQ values evaluated by different bioassays, were between 40 and 167% of the WHO-TEQ value). This must be considered as a relatively good agreement demonstrating the stability and reproducibility of the bioassay for use to analyze of biological samples. However, the TEQ value obtained from EROD micro-bioassay still being below the WHO-TEQ value, possibly, due to antagonistic effects of mono-or di-ortho PCB.

Therefore, the second round of intercalibration study attempted to clarify this hypothesis, the following mixtures are analyzed: PCDD/F + nonortho + PCB mixture and PCDD/F + nonortho + monoortho PCB mixture.

The results of different bioassays analysis are reported in (Fig. 5.5 and 5.6). The TEQ value obtained by micro EROD bioassay was also lower than this obtained by WHO-TEQ for the both of the mixtures. They represent 44 % in the case of PCDD/F + nonortho + PCB mixture and 30 % for PCDD/F + nonortho + PCB mixture, of the WHO-TEQ, demonstrating the antagonist of PCB, this effect is in this case strongly apparent for the non ortho than the mono ortho congeners.

Furthermore, the performances of different laboratories for analyze of environmental samples was examined during the first round of the intercalibration study, the test matrix was Fly ash. The fly ash sample was analyzed by 8 laboratories of which three used the CALUX assay. The DR-CALUX assay, the RTL-W1 bioassay, the H4IIE-luc, the MH1C1 EROD assay were performed by one laboratory each. The results of the fly ash analysis ranged between 32 and 2111 pg/g dry weight (Fig 5.3). The WHO-TEQ value was 2100 pg/g dry weight, which is a consensus value obtained from Dr. van Bavel's intercalibration study (the 7th round of the international intercalibration study on fly ash and soil/sediment samples). Two of the laboratories came relatively closer to this value (laboratory 3 and 4). Two other laboratories reported values that were around 30% of the WHO-TEF (lab 8 and 15), and the rest of the laboratories

reported values below 4% of the WHO-TEQ value. The TEQ measured obtained by micro-EROD bioassay was 54.8 ± 5.6 pg/g dry weight and present 3% of the WHO-TEQ value.

Fly ash is a difficult matrix, which contained elevated amounts of carbon, has a lower response in EROD bioassay; the discrepancy may be due to varying extraction efficiencies in the extraction procedures used by the different laboratories.

In order to avoid the extraction effects on the sensitivity comparison of bioassays, a fly ash extract was directly analyzed by 10 laboratories. The chemical fly ash analysis from 7th round of the international intercalibration study on fly ash and soil/sediment samples yielded a consensus value of 1060 pg/WHO-TEQs/g dry weight. The bioassay laboratories reported values ranging from 445 to 7400 pg TEQs/g (Fig. 5.4). Seven of the laboratories reported values above WHO-TEQ. None presented values that were below 40% of the WHO-TEQ value. Of the four laboratories reporting high values (above 3700 pg/g), three had not done any clean-up of the ash extract. This extract contained PAH, which probably contributed to the effect in these bioassays.

The RSDs ranged between 8 and 25% except for one outlier with a RSD of 75%. There is no overall difference in RSDs between the different bioassay types.

The micro EROD bioassay reported a value of 2333 ± 198 pg/g similar to this obtained by MH1C1 EROD assay (2198 ± 320 pg/g) and 20% higher than WHO-TEQ value, this may be due to the presence of other dioxin-like compounds such as polychlorinated naphthalenes and polybrominated dioxins/dibenzofurans in the extract. Thus, the EROD activity induction as endpoint can be also applied when testing complex extracts, provided that a PAH-removing clean-up step is included prior to testing.

In conclusion, in comparison with the other bioassays, micro EROD bioassay is able to predict the WHO-TEQ in biological and environmental samples fairly well. The extraction of fly ash sample is a critical step and requires more investigations. Furthermore the PCB congeners have an antagonist effect on the EROD activity induction in the presence of TCDDs.

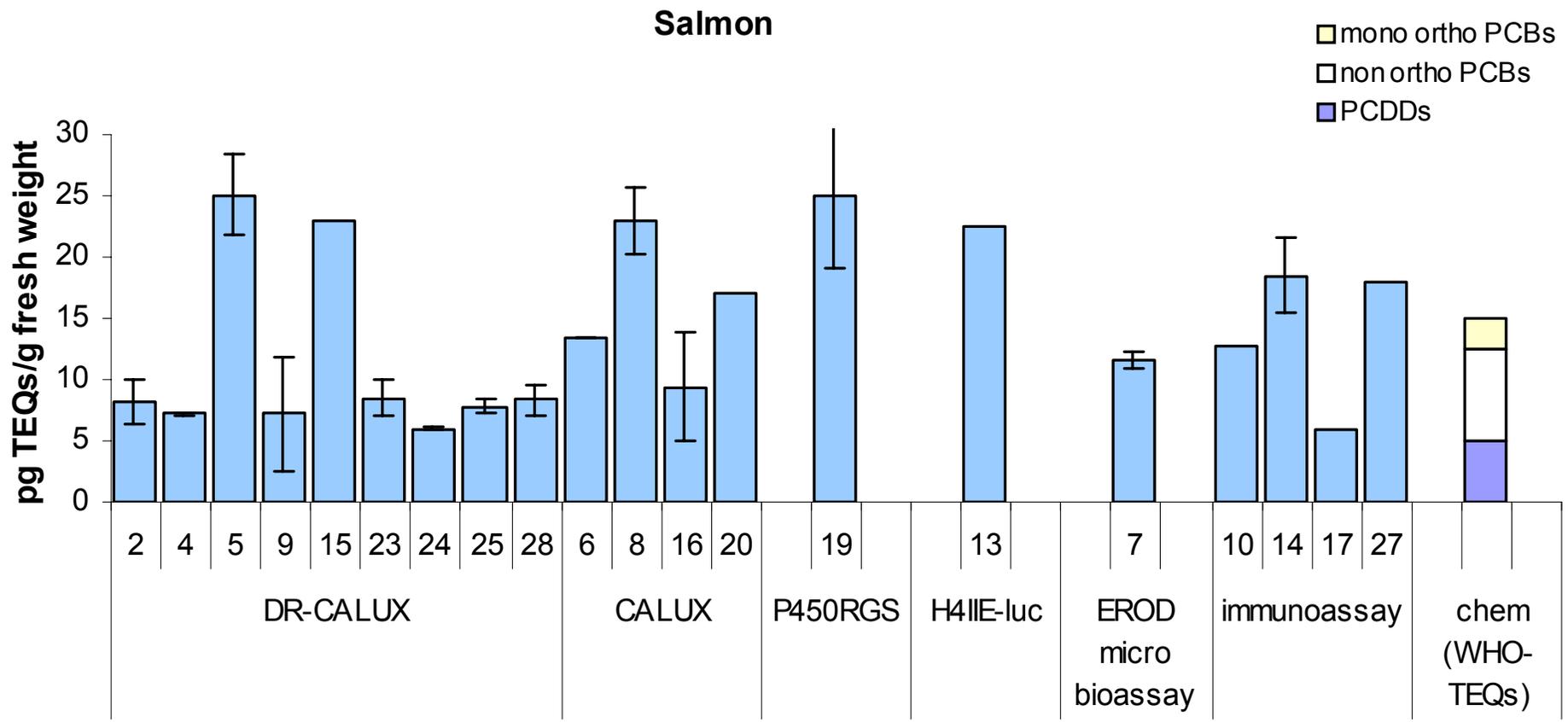


Fig. 5.1: Concentrations of dioxin-like compound in the salmon sample, determined by different bioassay laboratories and GC/MS analysis during the second round of the interlaboratory study comparison.

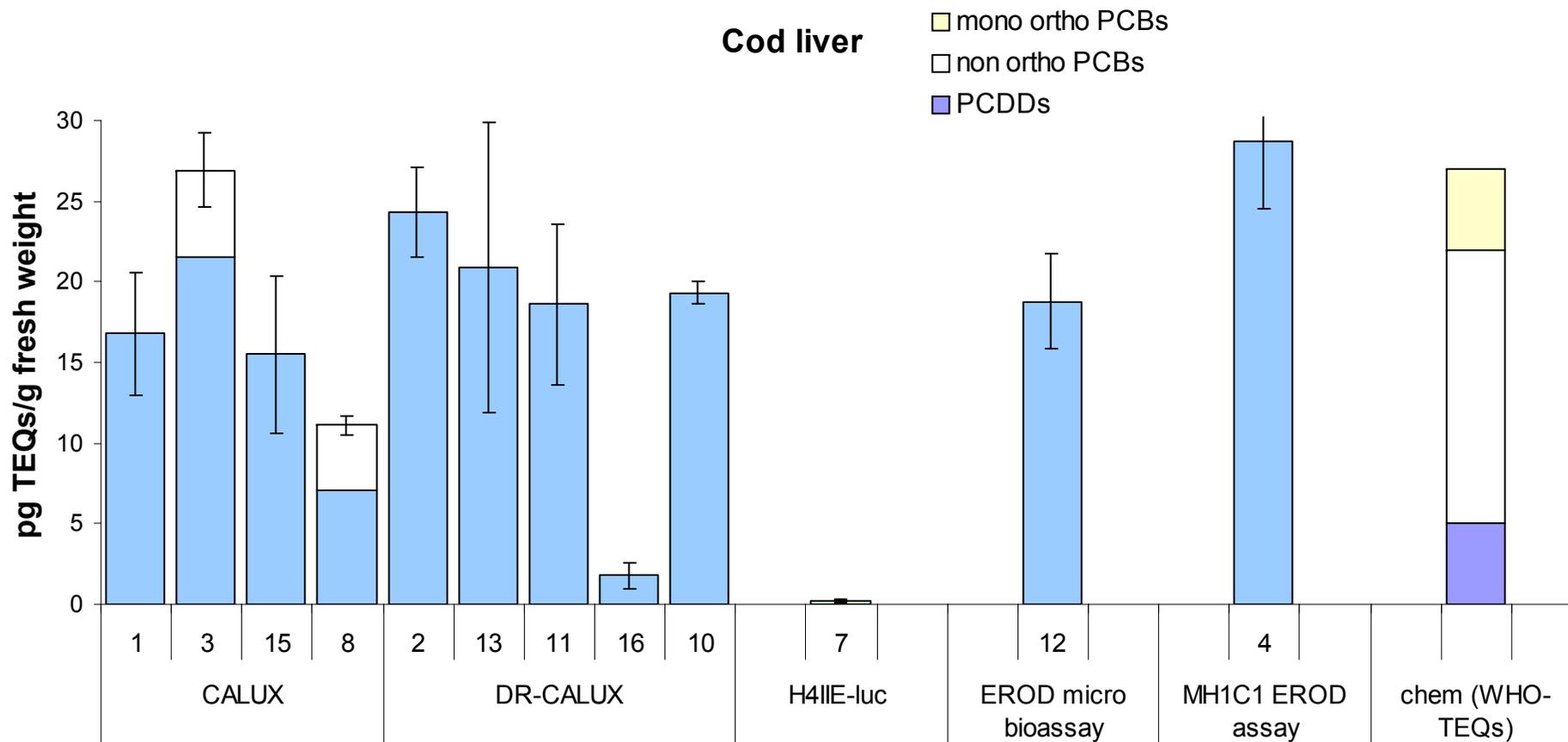


Fig. 5.2: Concentrations of dioxin-like compounds in cod liver sample, determined by different bioassay laboratories and GC/MS analysis during the first round of the interlaboratory study comparison.

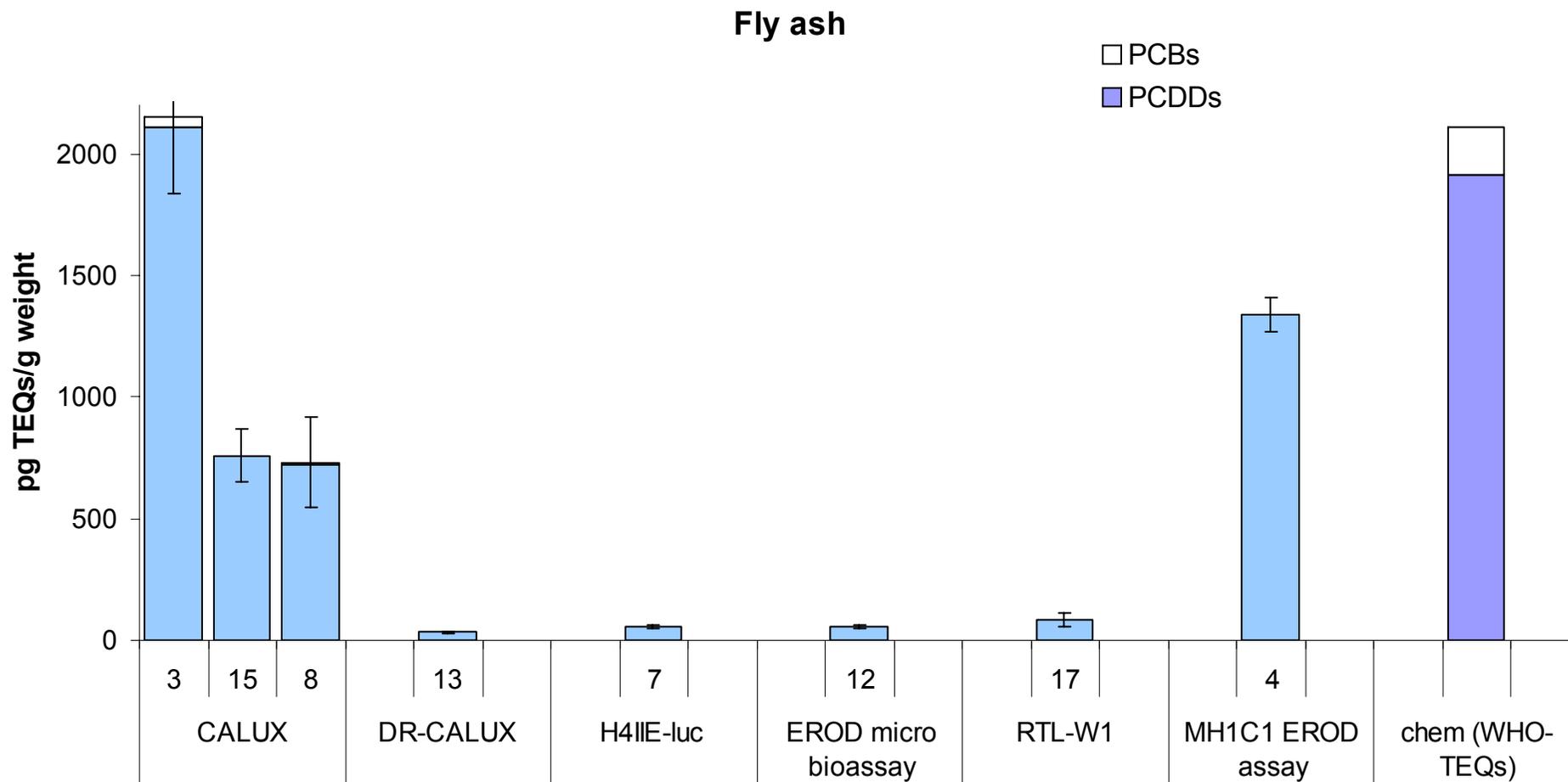


Fig. 5.3: Concentrations of dioxin-like compounds in the fly ash sample, determined by different bioassay laboratories and GC/MS analysis during the first round of the interlaboratory study comparison.

Fly ash extract

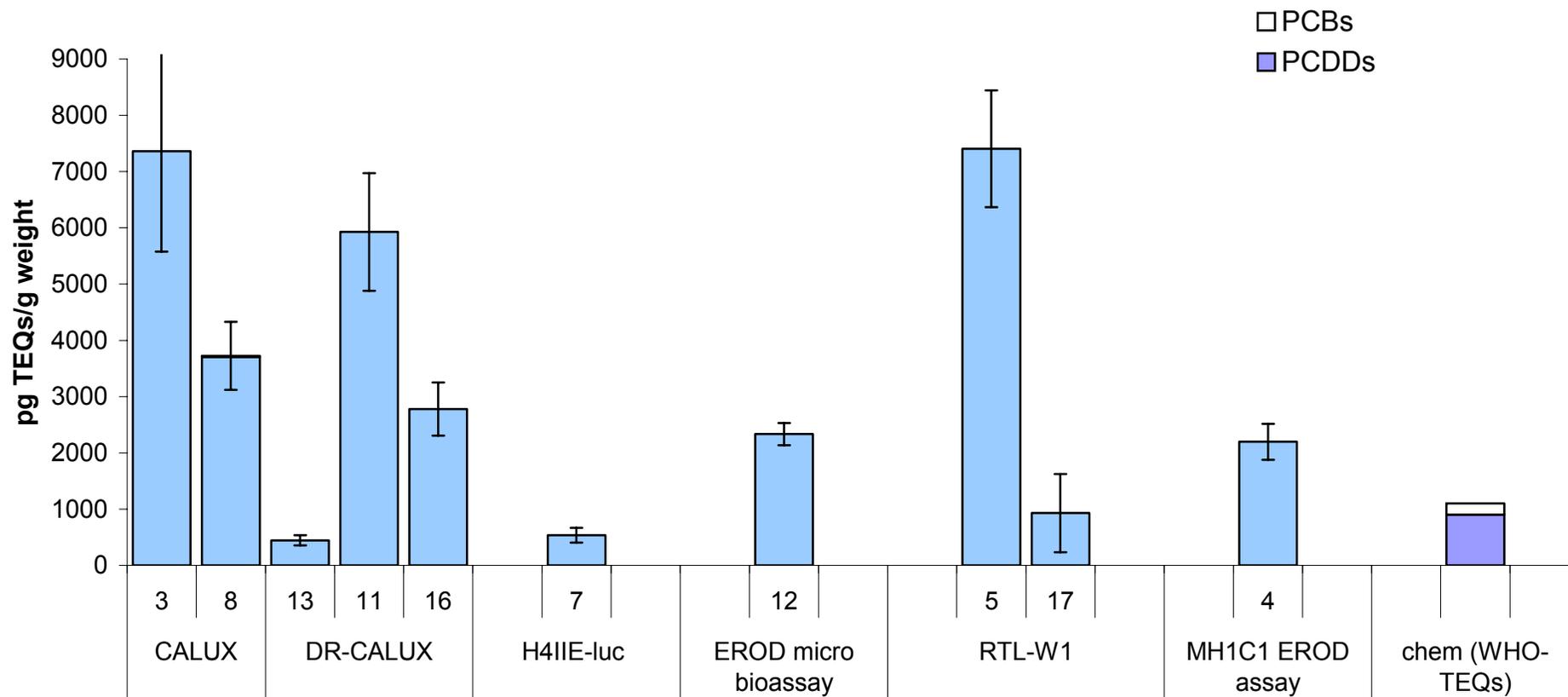


Fig. 5.4: Concentrations of dioxin-like compounds in the fly ash extract, determined by different bioassay laboratories and GC/MS analysis during the first round of the interlaboratory study comparison.

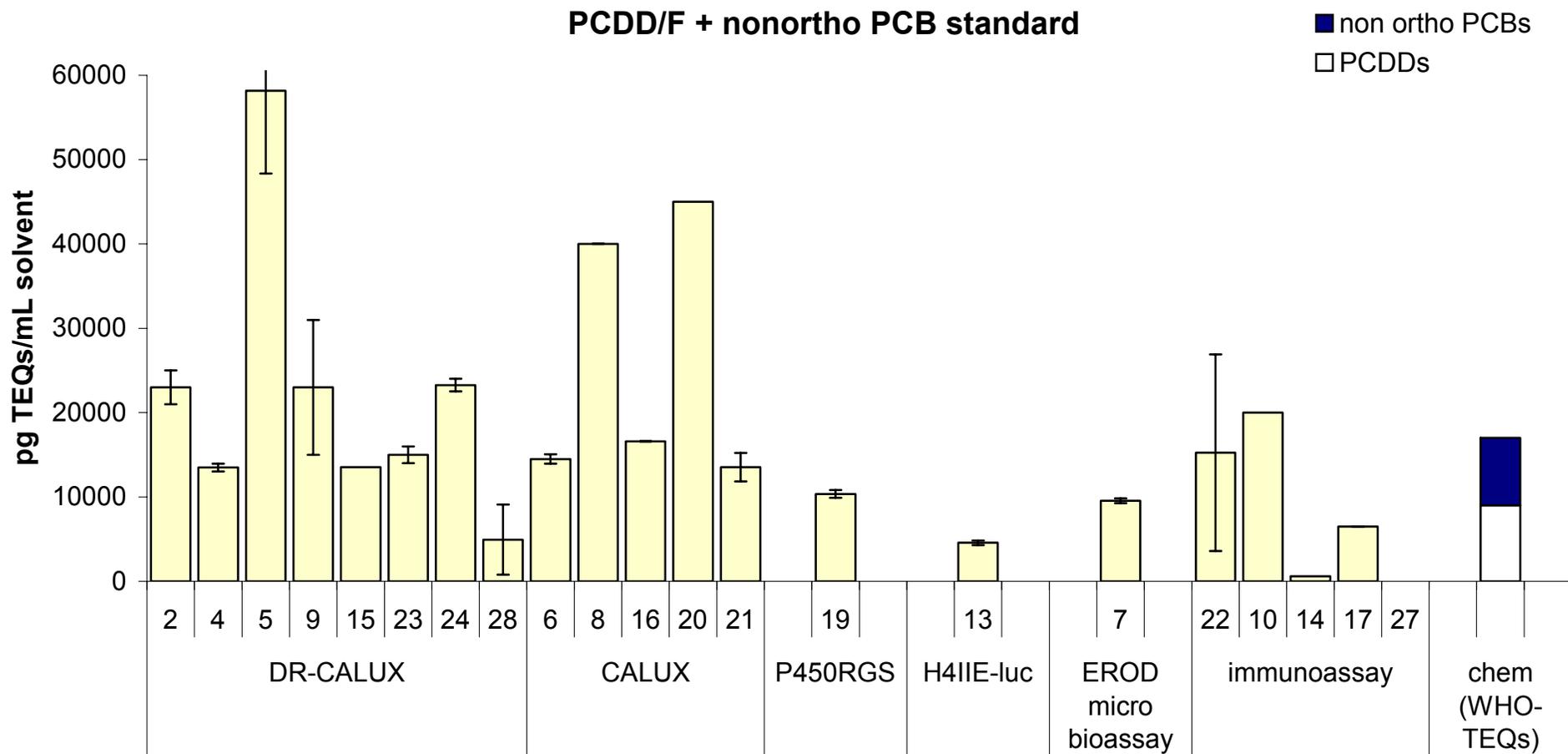


Fig. 5.5: Concentrations of dioxin-like compounds in (PCDD/F + non ortho PCB) standards mixture, determined by different bioassay laboratories and GC/MS analysis during the second round of the interlaboratory study comparison.

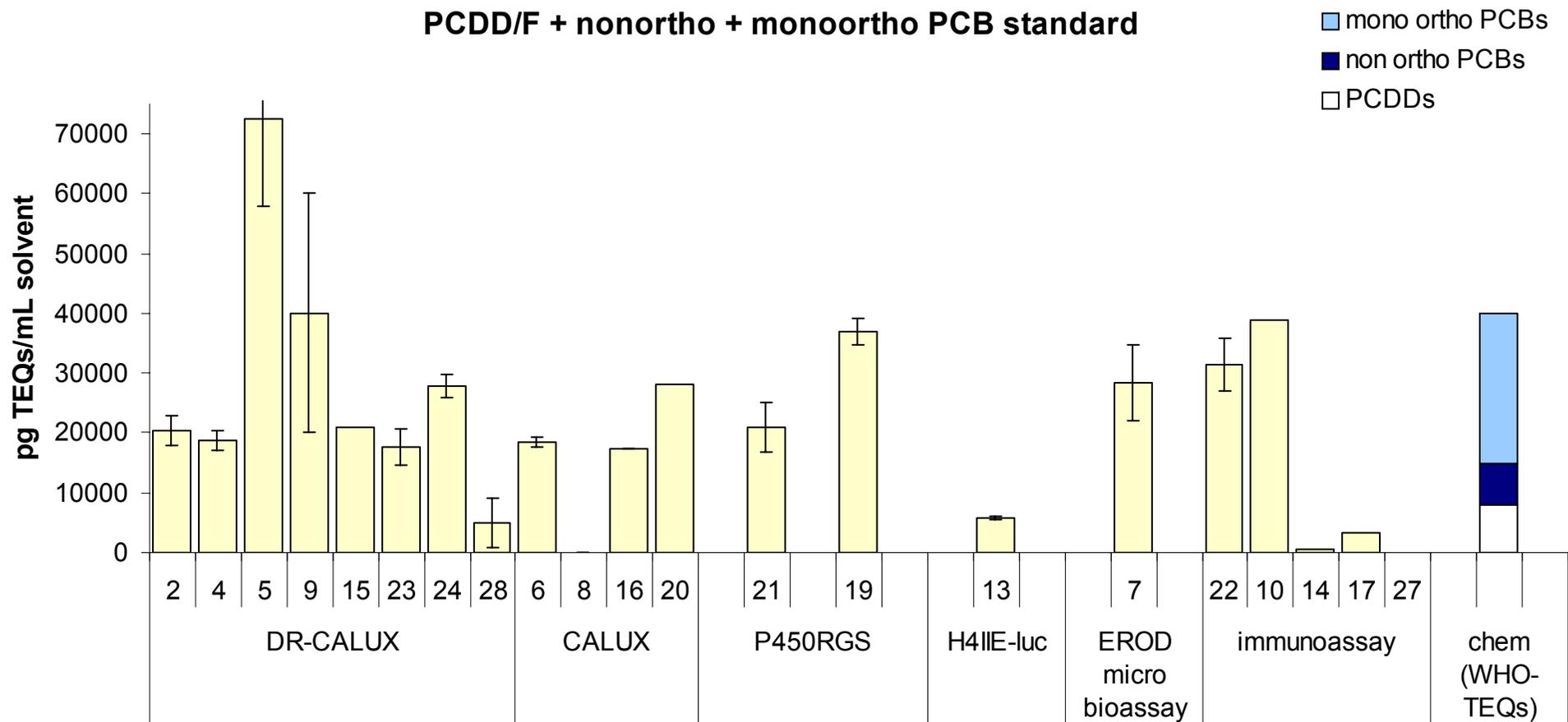


Fig. 5.6: Concentrations of dioxin-like compounds in (PCDD/F + non ortho + monoortho PCB) standards mixture, determined by different bioassay laboratories and GC/MS analysis during the second round of the interlaboratory study comparison.

5.3. Natural halogenated organic compounds

In this study, the fluorescence value was used directly to express the relative EROD induction level and compared with those of the standards curve of 2,3,7,8-TCDD (see experimental). The results showed that both Q1 and TBA at used concentration 15 µg/well (the highest concentration that could be tested) have a very low response: the fluorescence signal for TBA was 702.67 ± 12.83 (blank 689 ± 12.69) and for Q1 480 ± 12.69 (blank 443 ± 12.83). Related to EC50 value of 2, 3, 7, 8-TCDD (0.2 pg or 6.2.µM/well), both substances are in this test at least around the factor 7.5×10^7 less toxic (Fig. 5.7)

On the other hand, the physico-chemical parameters determination of Q1 and TBA reported by Vetter [143], agree well with other polyhalogenated bi-acrylic systems such as PCB, while aqueous solubility and vapor pressure of TBA (Table 5.2) was significantly higher however, the low water solubility of Q1 was, surprising, in view of the free electron pair on the two nitrogen's. And it was clear that the pyrrole units of Q1 cannot occupy planar conformation. Consistent with that, EROD induction potency and human Ah receptor binding, which typically (though not always) are a function of planarity, were low.

All observations are in agreement with the conclusion that, Q1 is not a planar molecule in all phases

Table 5.2: Physico-chemical parameters of Q1 and TBA

Compound	$S_{w,25}^{\circ C}$ (mol m ³) ^a	P (Pa) ^a	H (Pa m ³ mol ⁻¹)	Log Kow
Q1	2.38×10^{-4}	0.001683084	7.06	6.3
TBA	0.1519	0.065615696	0.4318	4.4

The results of other bioassays (ARH binding assay, Sulforhodamine B (SRB) assay Pesticide tests) may be interpreted in the way that Q1 is not acute toxic in comparison with known compounds which show these effects. It was also demonstrated that the effects of Q1 on typical pesticide test organisms (fungi, some plant bacteria, arthropods, and herbs) were negligible. This natural compound exhibited little biological activity in the assays employed. Thus, the role of Q1 in

nature is still mysterious. Pharmaceutical activity and other bioactivity need to be investigated as well, the (bio) metabolism of Q1 along with the toxic evaluation of potential metabolites should be explored. It cannot be excluded that initially formed metabolites may have effects on the systems applied. In light of the novel 1,2'-bipyrrole backbone, a prediction of the ecotoxicological fate in the environment is difficult. Similar work needs also to be carried out with TBA and other halogenated natural products.

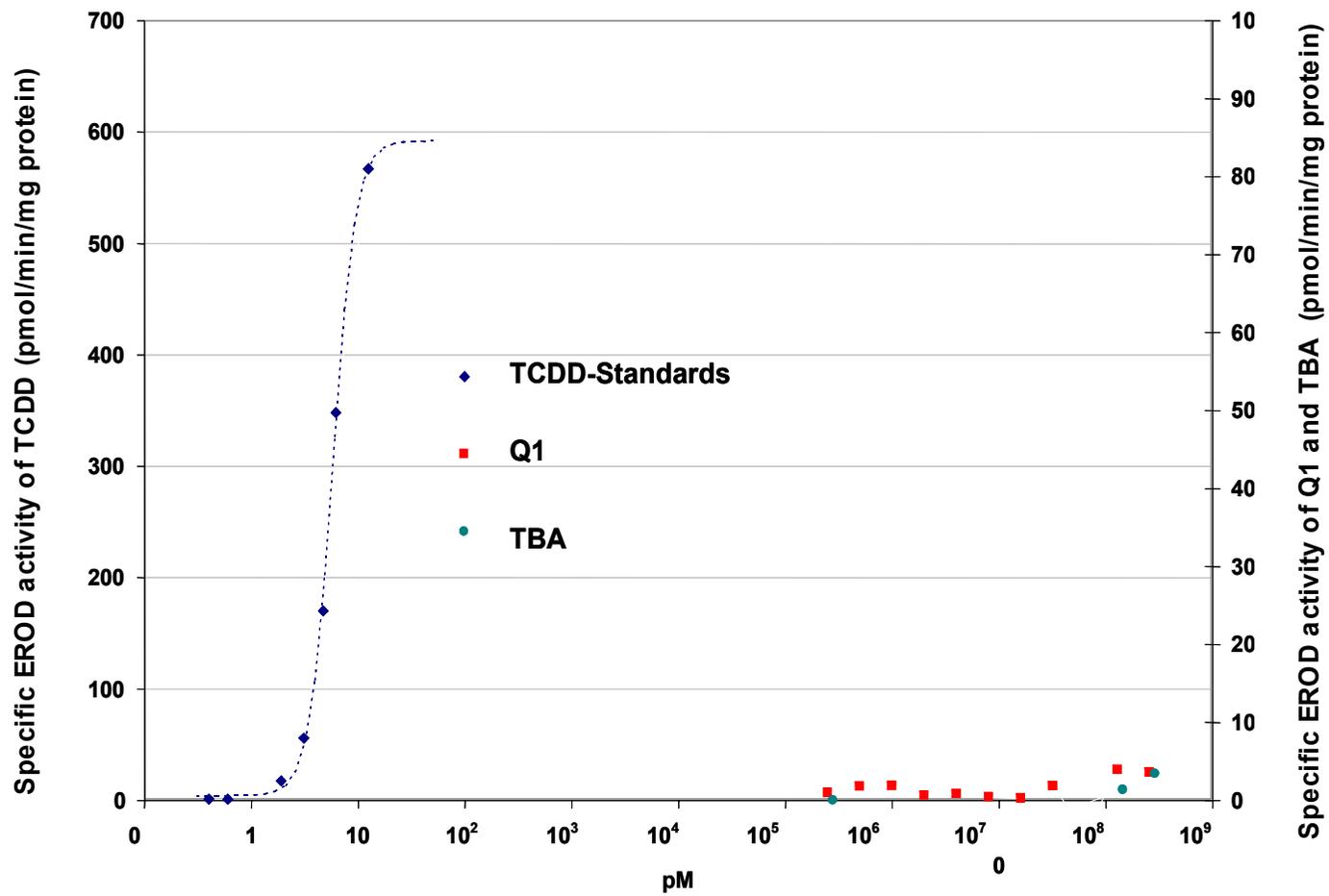


Fig. 5.7: Q1 and TBA induction in micro-EROD bioassay in comparison with standards response curve

5.4. Screening of dioxin– and estrogen like activity of leachate-polluted Groundwater

Leachate suspected polluted groundwater sample and reference commercial mineral sample (Staatlich Fachingen) as blank were investigated, employing two biological test systems, either for dioxin-like response or for estrogenicity.

In the EROD assay, the samples were tested as crude extract (without clean-up); in parallel employing a sample preparation (clean-up) where only the persistent dioxin-like compounds were detected. This assay showed a weak signal in the crude fraction, which corresponds to a concentration of 96pg TEQ/L, and 23pg TEQ/L in the fraction that contains the persistent dioxin-like compounds only.

The Yeast-recombinant assay was employed to test the raw water and extracts of the water prepared with n-hexane, dichloromethane, and benzene to cover different polarities of compounds. In this test the extracts of n-hexane and benzene were negative, whereas the raw water and the dichloromethane extract resulted in a positive weak signal, which corresponds to a water concentration in terms of 17- β -estradiol equivalent of 4.8 nM and 3 pM respectively. That means that the most estrogen-like compounds were present in the intermediate polarity. The same finding was reported by previous studies investigating the estrogenic activity of soot [144] and household stoves [145], after extraction with different solvents, the results showed that the largest fraction of the activity observed, was located in the medium polar dichloromethane eluate.

In conclusion the groundwater sample showed weak activities of estrogenicity and dioxin-like response. For further confirmation it is advisable, to concentrate more (100-1000 L) and to repeat the test with higher pre-concentration of the water by employing C18 and/or XAD resins as well as freeze drying step for the raw water.

Table 5.3: compilation of EROD assay results for water sample

TEQ (pg/l)	Assay 1	Assay 2	mean	standard deviation
Without clean up	91.49	100.09	95.79	4.30
With clean-up	10.64	36.16	23.40	18.04

Table 5.4: Yeast assay results of water sample with extraction

Absorbance 550nm-620nm - Response 72 h								
Concentration μL sample/well extraction	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean	Standard deviation	Solvent used for the extraction	β-galactosidase activity expressed as 17β-estradiol nM in the sample
5000	-0.003	-0.007	-0.005	-0.004	-0.004	0.001	Benzene	No detectable
5000	-0.024	-0.013	-0.014	-0.011	-0.012	0.001	Hexane	No detectable
5000	0.116	0.078	0.084	0.111	0.104	0.014	CH ₂ Cl ₂	0.003
2500	-0.004	0.004	0.004	0.000	0.003	0.002	Benzene	No detectable
2500	-0.011	-0.005	-0.004	-0.001	-0.003	0.002	Hexane	No detectable
2500	-0.006	0.001	-0.003	-0.005	-0.004	0.001	CH ₂ Cl ₂	No detectable

Table 5.5: Yeast assay results of water sample without extraction

Absorbance 550nm-620nm - Response 72 h							
Concentration μL sample/well	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean	Standard deviation	β-galactosidase activity expressed as 17β-estradiol nM in the water sample
2	-0.011	-0.012	0.000	-0.008	-0.010	0.002	No detectable
2	0.000	-0.003	-0.012	-0.012	-0.001	0.003	No detectable

Table 5.6: Yeast assay results of the mineral water blanks extracted with different solvents

Absorbance 550nm-620nm - Response 72 h							
Concentration μL water/well	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean	Standard deviation	Extraction Solvent
5000	-0.008	-0.002	-0.002	0.000	-0.001	0.001	Benzene
5000	-0.005	-0.002	-0.004	-0.004	-0.004	0.000	Hexane
5000	-0.005	0.001	-0.002	-0.007	-0.004	0.002	CH ₂ Cl ₂

5.5. Bio-monitoring of dioxin-like compounds in mother milk

5.5.1. Dioxin-like compounds content in breast milk

The screening of dioxin-like compounds in mother milk samples was performed in two steps. For the first step the Danish samples analysis was carried out following the standard protocol, the results showed no EROD activity induction below the operating conditions (data not shown), this negative responses was attributed to the detection limit of the assay method. In order to improve the sensitivity of the Screening method, a modification of the standard protocol was required and the samples were more concentrated, that means after clean-up procedure the samples were transferred in 25 μL DMSO/Isopropanol 4:1 (v/v) instead 500 μL in the standard protocol. All the analyzed samples were found to contain detectable dioxin-like activities using the H4IIE cell EROD screening assay.

26 breast milk samples of Denmark were analyzed; the mean EROD-TEQ values were 0.47 ± 0.33 pg/g of milk weight and 18.79 ± 16.12 pg/g of milk fat. EROD-TEQ values of different samples varied from 0.21 to 1.54 pg/g of milk weight and 5.55 to 87.98 pg/g of milk fat. Moreover, a relatively high standard deviation was noticed 16 % and was be due to the presence of one sample (code 1020) that present TEQ value far higher than others (88 pg/g lipids), on a whole weight basis this sample showed also a high TEQ value (1.54 pg/g weight milk). On the other hand this sample has elevated fat content 1.75 g which tends to increase the lipid correlated value. For reasons of homogeneity, we did not incorporate this “out of range” sample in mean calculation. For this group of samples, the new mean TEQ value is 16 ± 8

pg/g of milk fat, and they presented levels good distributed around the mean and still included in a 95% confidence interval, as illustrated in (Fig. 5.8)

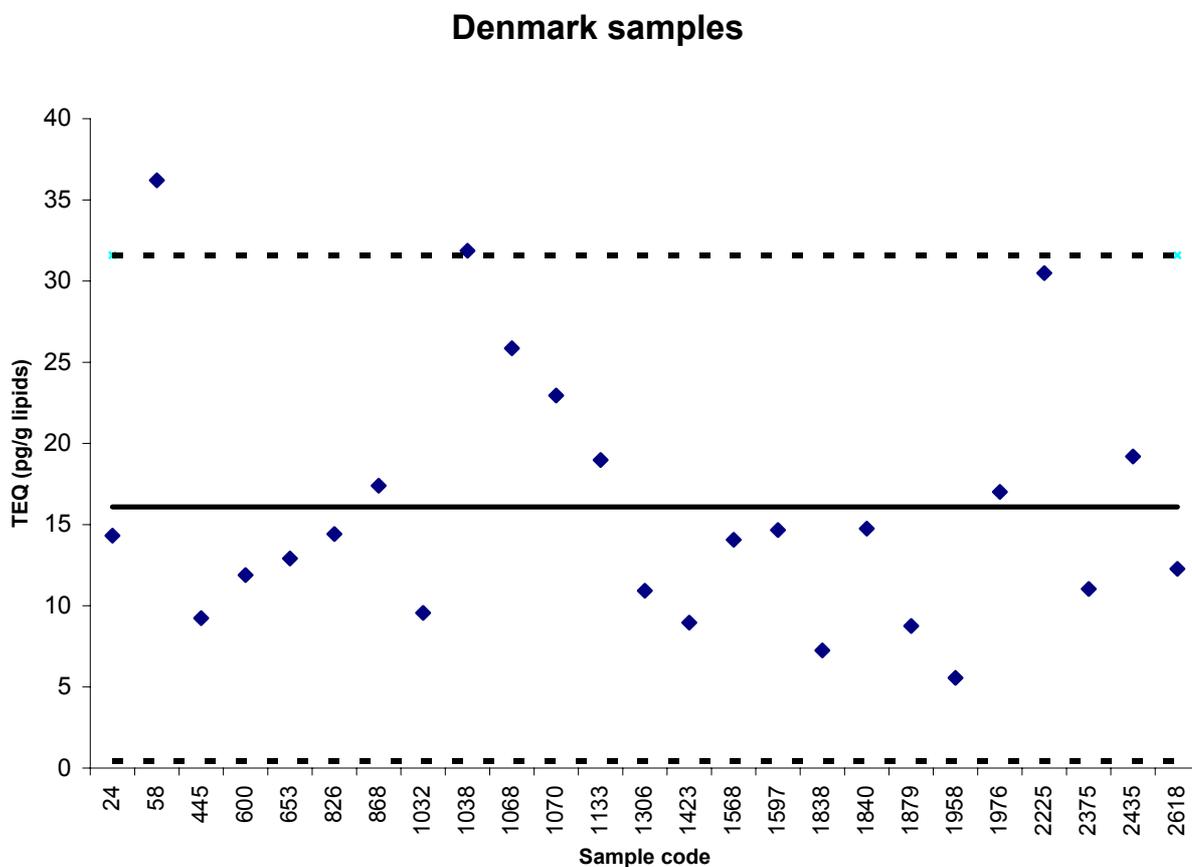


Fig. 5.8: Distribution of Denmark sample concentrations. Central line represents the mean value, while top and bottom dotted lines delimit 95% confidence interval

For 18 breast milk analyzed samples from Finland, the obtained TEQ value are very low than those from Denmark. The mean EROD-TEQ values, was 0.33 ± 0.51 pg/g of milk weight and 8.76 ± 14.03 pg/g of milk fat. EROD-TEQ values of different samples varied from 0.03 to 1.91 pg/g of milk weight and 0.69 to 46.88 pg/g of milk fat.

Similar to Denmark samples, among analyzed samples, two samples presented high TEQ values than the other and situated over of the 95% confidence interval (46.24 and 46.88 pg/g of milk fat), this samples showed also a high values on the basis on the whole milk weight (1.46 and 1.91 respectively).

Excluding these “out of range” samples, the Finland samples presented a TEQ mean value of 4 ± 2.8 pg/g of milk fat, and are regularly distributed around of this median, in the 95% confidence interval (Fig. 5.9).

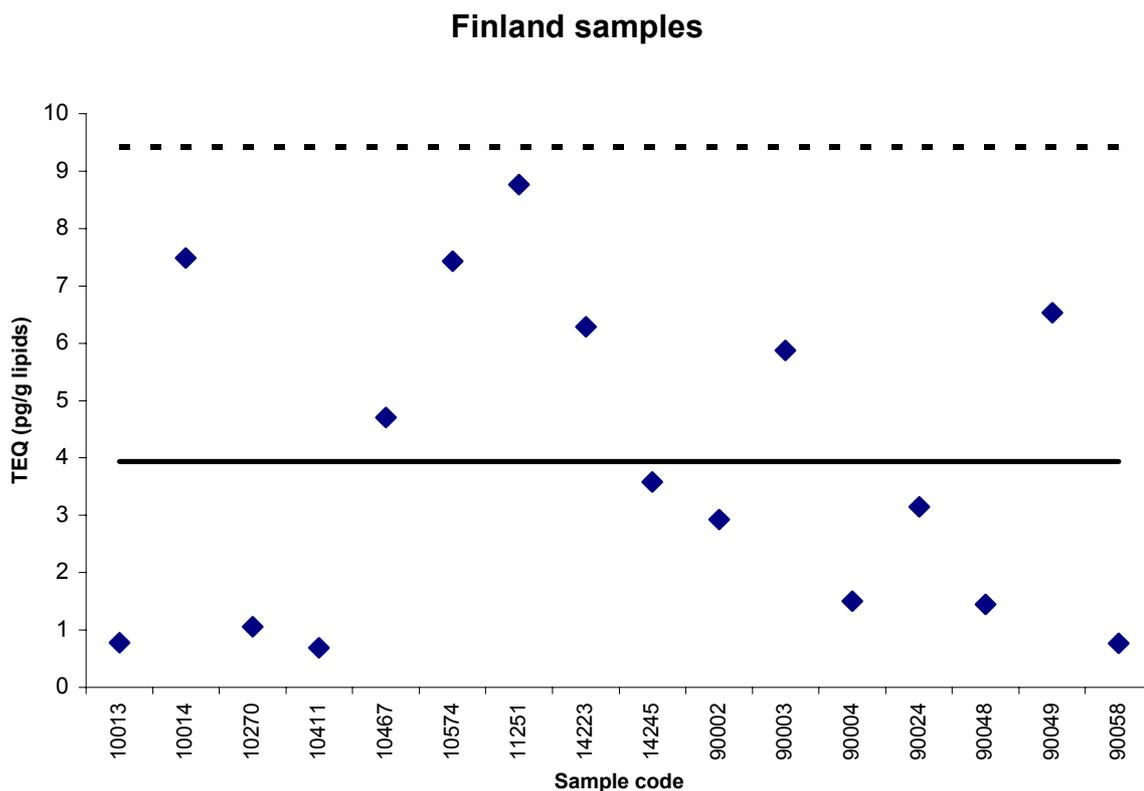


Fig. 5.9: Distribution of Finland sample concentrations. Central line represents the mean value, while top and bottom dotted lines delimit 95% confidence interval.

The results of screening of halogenated hydrocarbons and chiral persistent bioaccumulating endocrine disrupting chemicals in these samples, investigated by Shen [134], reported that the anti-androgenic p,p'-DDE was dominant pollutant in all samples. Other major pollutants were β -HCH, HeCB, END-1, dieldrin, OXC, c-HE and p,p'-DDT, which were all weak Endocrine disrupter. The total amount of the 8 compounds changed from 42.93 to 524.49 ng/g. Samples from Denmark contained a higher level of investigated pollutants in placenta (1.73 times) and milk (1.82 times) This results correlate with those obtained in this study concerning dioxin-like compounds contamination, which emphasizes the more higher contaminated samples in Denmark cohort that in Finland ones.

5.5.2. Relationship between dioxin-like compounds and lipids content

The lipid content is generally an important factor for the accumulation of dioxin-like compounds in mother milk, due to their high lipophilicity. Therefore, an investigation is

performed to attempt, if there is any correlation between the dioxin-like compounds and lipids content in the analyzed samples.

Figure 5.10 shows the relationship of the dioxin-like compounds content in TEQ to the content in Danish milk. The lipid content was 1.15 to 6.1%. Exclusive two samples, a probable correlation was indicated, the same phenomena was registered by the finish samples (1.38 to 6.1 % lipid content).

PCDD and PCDF are dispersed equally among in the lipid compartments of breast milk [146]. A higher lipid contents (2-7 %), on the contrary is negatively correlated with the dioxin content in total lipids of breast milk [147]. This could be due to a diluting effect of dioxins by lipids. However, our results did not show such diluting effects, and we proved relatively constant TEQ-based levels, expect for the 2 abnormally dioxin-like compounds abundant mother milk specimens of Denmark and Finland. In these cases, we must take notice that the total amount of dioxins in breast milk increased slightly as the lipid content increased. The same finding was obtained by Sampei [148] when analyzing dioxins in breast milk of primiparas in the Yonago district in Japan.

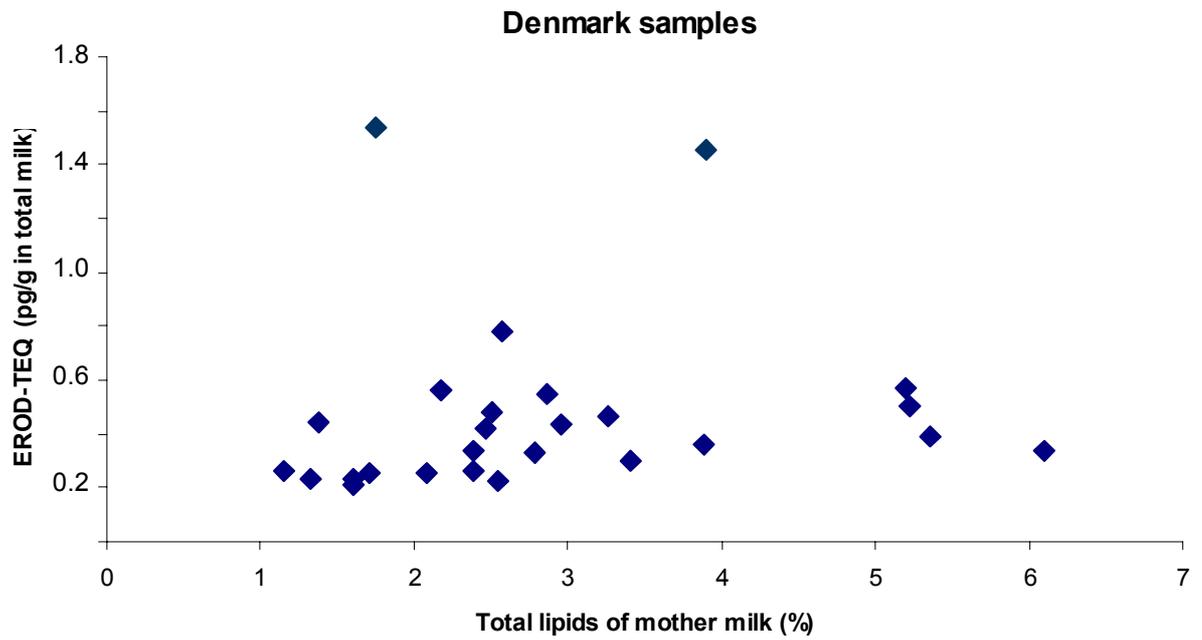


Fig. 5.10: Relationship between the total lipid and dioxin-like compounds contents in Finnish mother milk

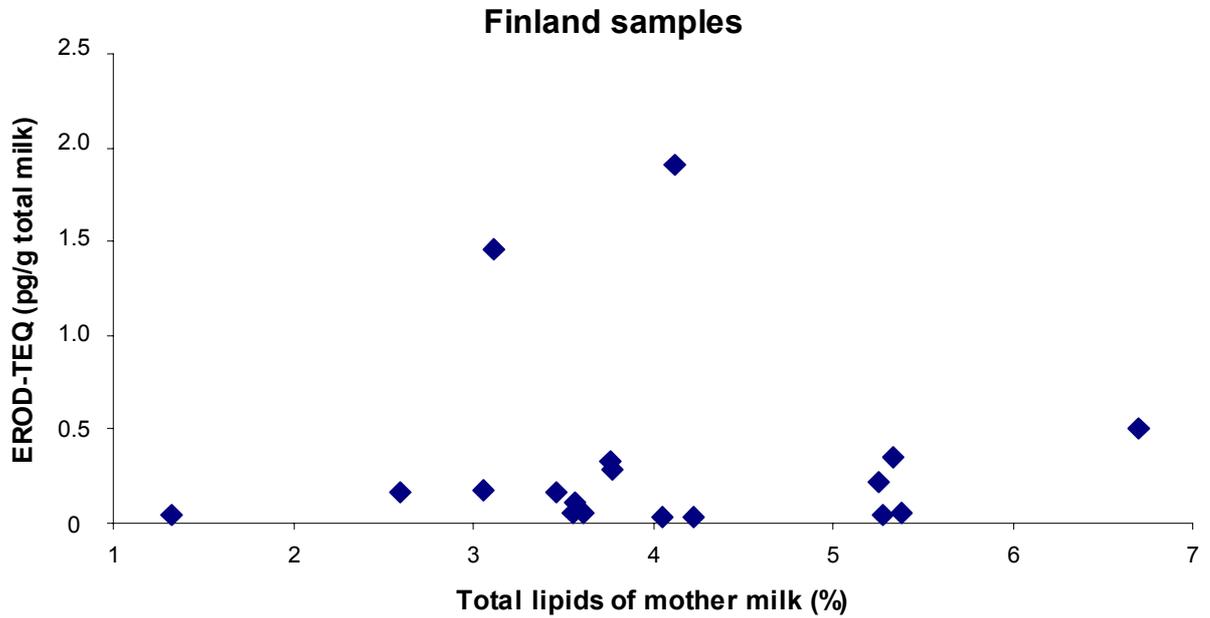


Fig. 5.11: Relationship between the total lipid and dioxin-like compounds contents in Finnish mother milk

5.5.3. Correlation analysis between micro EROD bioassay and chemical analysis data

The chemical analysis data of the investigated samples indicated that the mean chemical -TEQ values of the 26 samples from Denmark ranged from 8 to 49.2 pg/g of milk fat while the 18 samples from Finland showed mean values of 9.2 to 41.9 pg/g of milk fat. These levels would be within the range of contamination reported, and were comparable to those of other countries. For example the detectable dioxin concentrations, in terms of chemical-TEQ (C-TEQ), have been as following: 9.6–35 pg/g fat (PCDD/F) in Sweden [149], 9.9–48.5 pg/g fat (PCDD/PCDF/CoPCB) in Japan [150], 16–40.2 pg/g fat (PCDD/F) in the Republic of Uzbekistan [151], 21–53 pg/g fat (PCDD/F) in agricultural regions of southern Kazakhstan [152] and 5.9–17.1 pg/g fat (PCDD/F) in Spain [153]. LaKind [154] reported a review of world-wide data on C-TEQs (PCDD/F) in breast milk. During the years 1970–1996, the world-wide reported C-TEQ values were in the range of 3.1–484 pg/g fat. The highest value was reported in Vietnam in 1970, mainly due to the spraying of Agent Orange during the Vietnam War.

The relationship between the chemical analysis and micro-EROD bioassay data was examined (Fig. 5.12, Fig. 5.13). For both cases it was clearly that the TEQ value reported for EROD bioassay are lower than those of chemical analysis, in exception of the out of confidential range samples mentioned above. They present too high levels in comparison with chemical TEQ, that means that these values are not true and may be resulted by an experiment error during the bioassay analysis.

The comparison of the results is summarized in Table 5.6 in term of mean TEQ, the out of range sample are excluded from the calculations. It was reported that the difference between EROD and chemical TEQ of Finnish samples is high than for Danish sample, that suggests the presence of antagonistic compounds in samples mixtures that suppressed the level of EROD –TEQ.

Table 5.7: Comparison of EROD- and chemical TEQs

	EROD- TEQ (pg/g milk fat)	C- TEQ (pg/g milk fat)
Denmark samples	16 ± 7.92	23.4 ±10
Finland samples	3.9±2.8	19.32±9.8

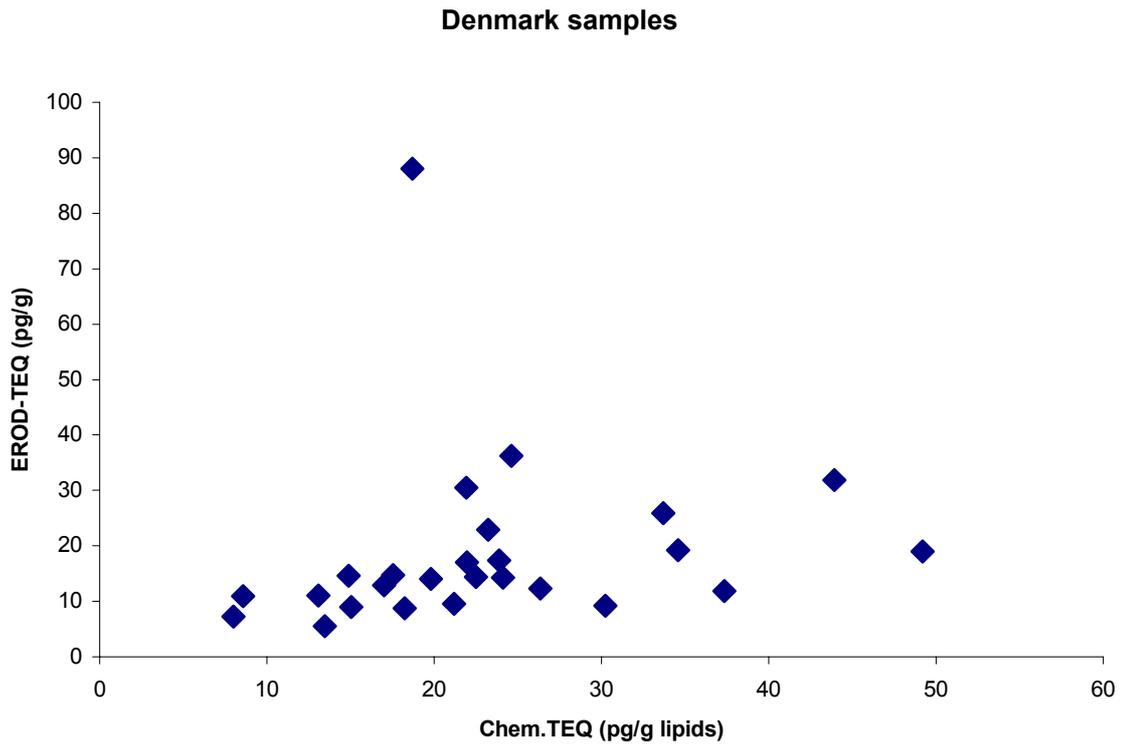


Fig. 5.12: Relationship between EROD- and Chemical TEQ in the case of Denmark samples mother milk

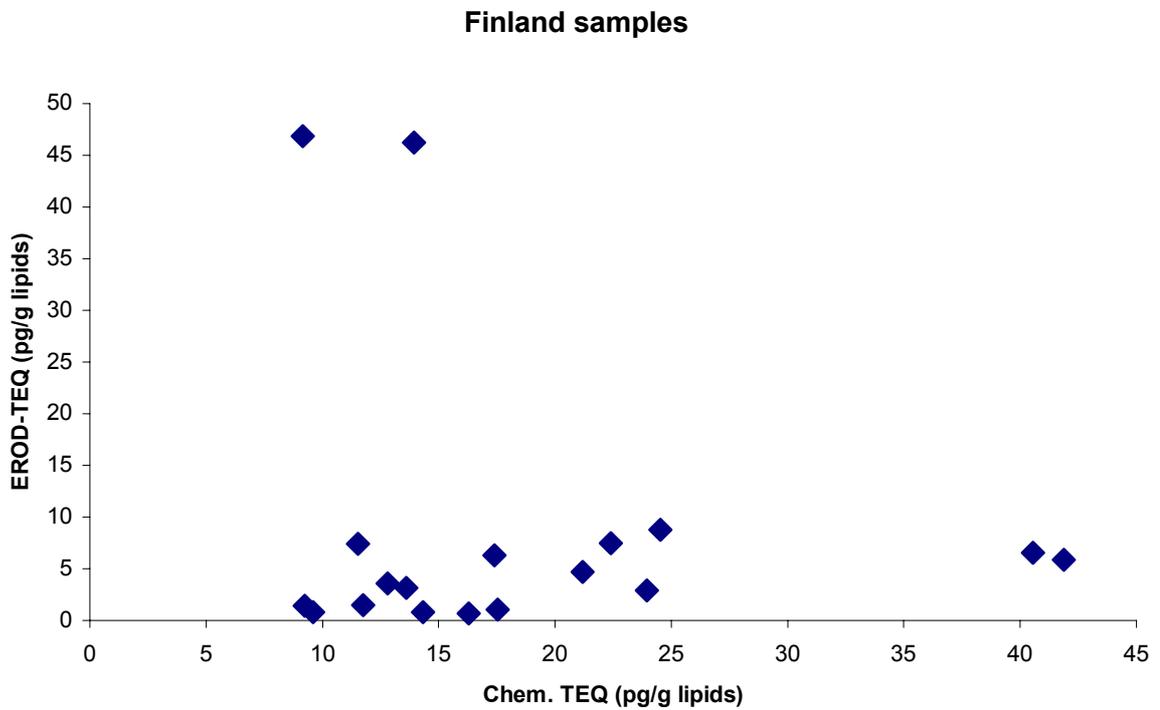


Fig. 5.13: Relationship between EROD- and Chemical TEQ in the case of Finnish mother milk

Furthermore, regarding a distinct endpoint, a result from biological assay can be compared with the information governed from instrumental analytical techniques some special cases information about the exposure of a single chemical within a mixture is known and furthermore the effect of this mixture can be described by a toxicological effect model.

If we define the ratio (R_{ba}) between bioanalytical and chemoanalytical response as:

$$R_{ba} = B/A$$

Where, B is the biological response value related to a comparable response considered by the toxicological model for A. Any response is actually based on the effective amount (Mol) of single compounds. This amount is normally related to the matrices gas, liquid and solid with their unit's m^3 , L and kg, respectively.

Taking concentration additivity into account the value of R_{ba} should meet unity if the response can be explained by A. If not, R_{ba} should exceed unity in any case as far as concentration additivity is valid. Then large values of R_{ba} can be attributed to the presence of additional chemicals with toxicological impact, and more investigations are required [48].

In our study, we tried to examine the effect of PCDD/F and PCB content on the correlation between EROD bioassay and chemical TEQ data. The level of PCB content in Denmark sample ranged from 34.2 to 61.9 (42.21 ± 5.7 pg/g fat) (Fig. 5.14) and from 31- to 51 pg/g fat for samples from Finland (39.4 ± 5.6 pg/g) (Fig 5.16), the mean of PCB content is similar in the both cases.

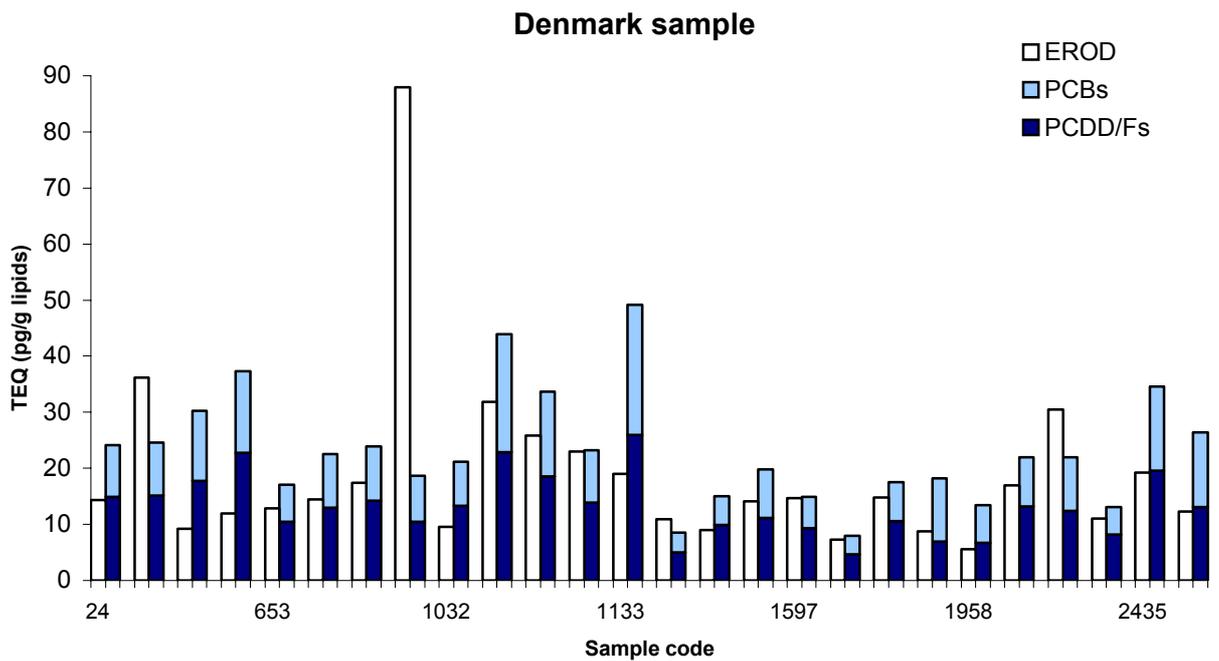


Fig. 5.14: Comparison of dioxin-like compounds with PCDD/F and PCB in samples from Denmark.

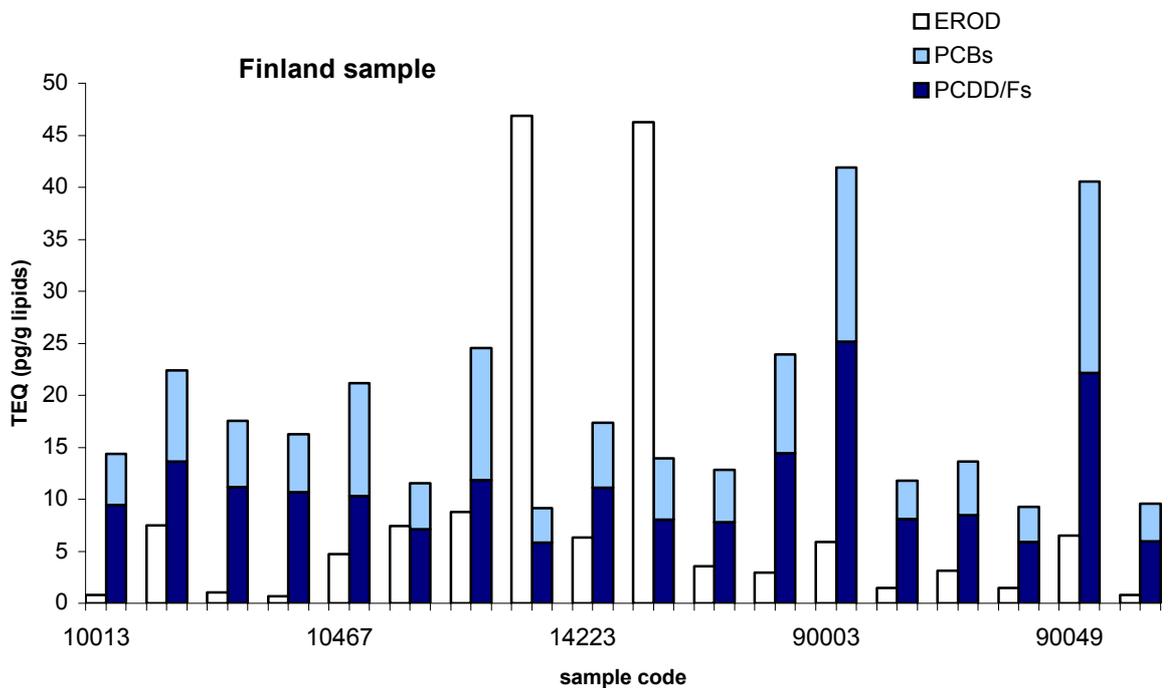


Fig. 5.15: Comparison of dioxin-like compounds with PCDD/F and PCB in samples from Finland.

To verify the effect of PCB and PCDD/F concentration on the correlation between Chemical analysis and EROD Bioassay, we plotted the content of these compounds again the Rba ratio. (Fig. 5.16 and Fig. 5.17), for the samples from Denmark the Rba values, are situated between and 0.3 and 1.46; 85 % of samples are situated below 1, and present a mean value of 0.73 ± 0.31 . In this case, it is shown, that the Rba values correlate positively with PCDD/F, but negatively with PCB contents, indicating the antagonistic effect of PCB in the biological Bioassay, the similar observation was reported in the results of Intercalibration on laboratory study for biological samples (salmon and cod liver).

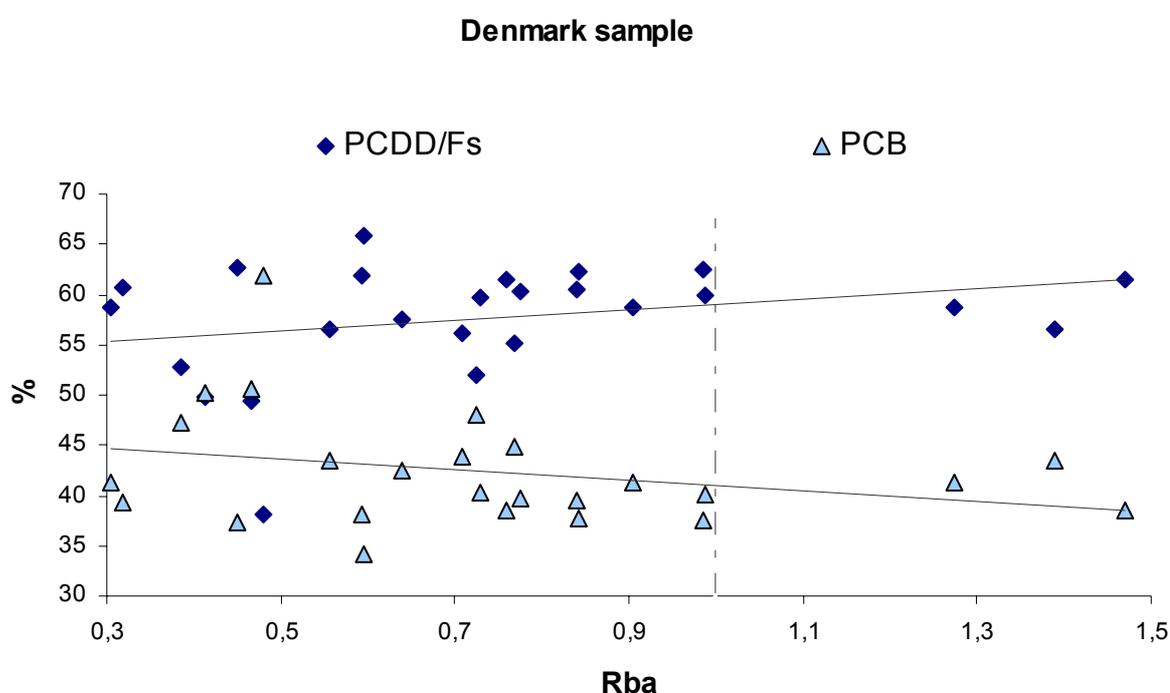


Fig. 5. 16: Relationship between PCDD/F and PCB content in the sample with Rba ratio in Denmark mother milk.

On the other hand, surprising results were registered for samples of Finland. Where the Rba values are very low, ranged from 0.05 to 0.64, and present a mean value of 0.73 ± 0.31 . In this case the correlation of Rba is in contrary of the Denmark samples positively with PCB and negatively with PCDD/F content, may be due to the presence and interaction of other antagonist compound complexes with Ah receptor in the mixture samples.

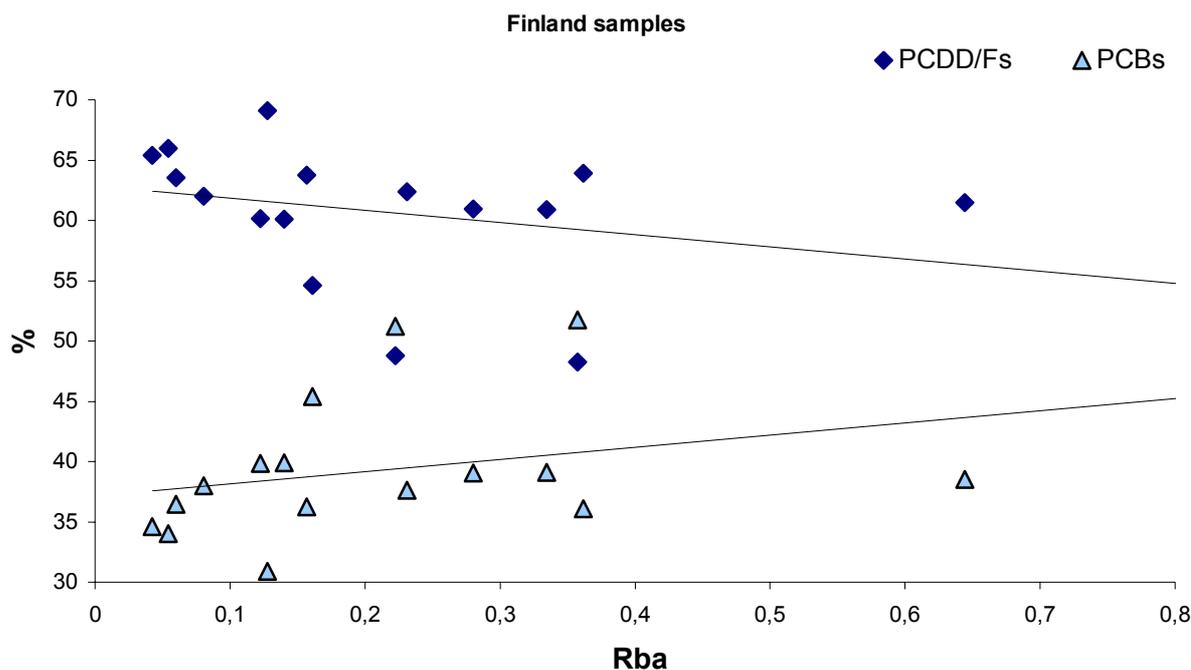


Fig. 5.17: Relationship between PCDD/F and PCB content in the sample with Rba ratio in Finnish mother milk.

Despite the results indicated above, EROD-TEQ and Chemical TEQ analyses have particular pros and cons, and thus caution should be taken in when interpreting the data. It was understandable that EROD-TEQ detected the interaction of all AhR agonists, including both identified and unknown species. On the contrary, the C-TEQ approach detects all AhR agonists and thus by itself is incomplete. Chemical analysis indicated the type of contaminants that could be transferred to newborns during breast-feeding; however, this is not indicative of the biological or toxicological consequences of their exposure. In addition, because different studies adopt a variety of methods, different C-TEQ data are not always comparable. Although there was a very good correlation between EROD-TEQ and C-TEQ, for further improvement of the EROD-TEQ method, a more practical consideration of the complex interactions of the AhR agonists and antagonists present in sample mixtures should be determined. This could strengthen and increase the reliability of EROD-TEQ analysis. Although a number of studies have indicated that both dioxin-like and non-dioxin-like compounds might cause similar toxic effects through different mechanisms [155-158]. The present study using the EROD-TEQ assay did not take into consideration the possible risks imposed by non-dioxin-like compounds such as p,p'-DDT and p,p'-DDE. In addition, the methods utilized in this study cannot

distinguish among chemicals that may include other toxic agents contaminating the breast milk samples. However, some of them have already been addressed in a previous study [134]. Nevertheless, our results indicate that the EROD-TEQ assay was a very sensitive screening tool and is therefore particularly valuable to a cost conscious, high through put breast milk-monitoring program.

5.6. Metabolic activation of Bisphenol A for Estrogenicity screening

5.6.1. Optimization of Yeast assay

The first step to develop a metabolic activation protocol is optimizing the yeast assay to make it compatible with the bioactivation step with H4IIE. With increased incubation time from 24h to 48 h, the 17β -E2 dose–response curve shifted to the left, and hence the yeast assay became more sensitive (Fig. 5.18). The concentration of 17β -E2 required to produce half the maximal response is reduced. A prolongation of incubation time to 72h present no significant effect, and 17β -E2 produce the same submaximal dose–response curves as already obtained for 48h. The response curve of 17β -E2 in yeast bioassay after protocol optimization is shown in Fig. 5.18.

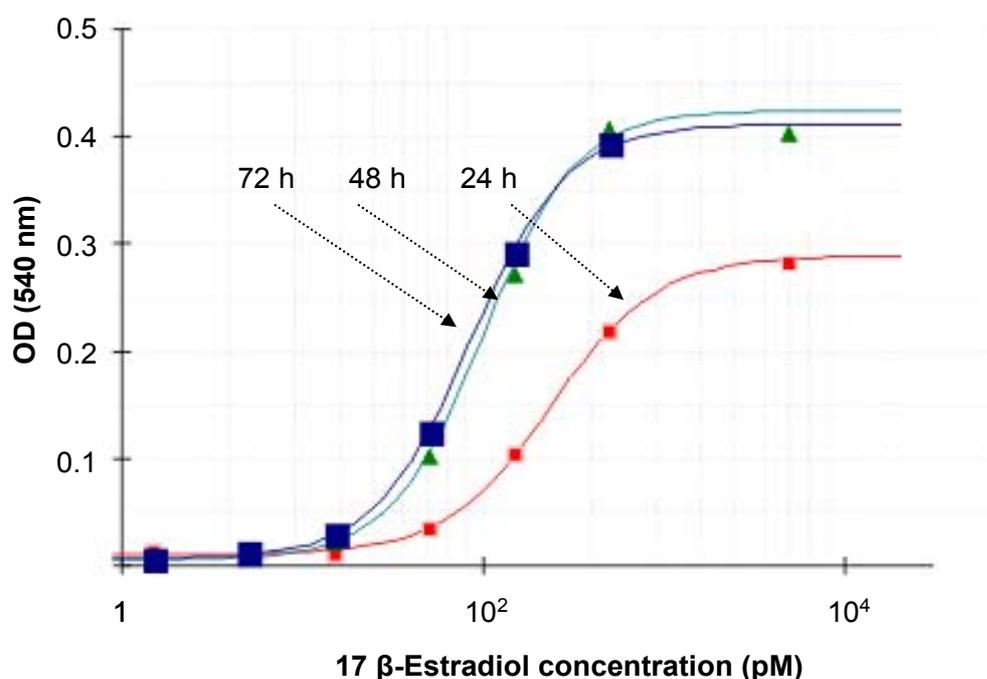


Fig. 5.18: Dose response of 17β -estradiol in yeast assay, the effect of the incubation time.

The performed modification in yeast assay, are summarized in table 5.7.

Table 5.8: Performed modifications in standard protocol in yeast assay

	Standard assay	Modified assay
Used solvent	Ethanol	DMSO/Isopropanol (4:1)
Mode of addition of test chemical	The solvent is evaporated before the addition of the medium.	The chemical was added directly to the medium
Incubation time	72 h	48h

Finally Bisphenol A estrogenicity was determined by yeast modified protocol (Fig. 5.19), BPA exhibits weak estrogenic activity, the binding of BPA to the estrogen receptor and present 10,000 times less than that of 17-estradiol activity, in consistence with the results reported by other investigations using other estrogen screening protocol [114], [117]. That means that the performed modifications did not affect the performance of the yeast assay.

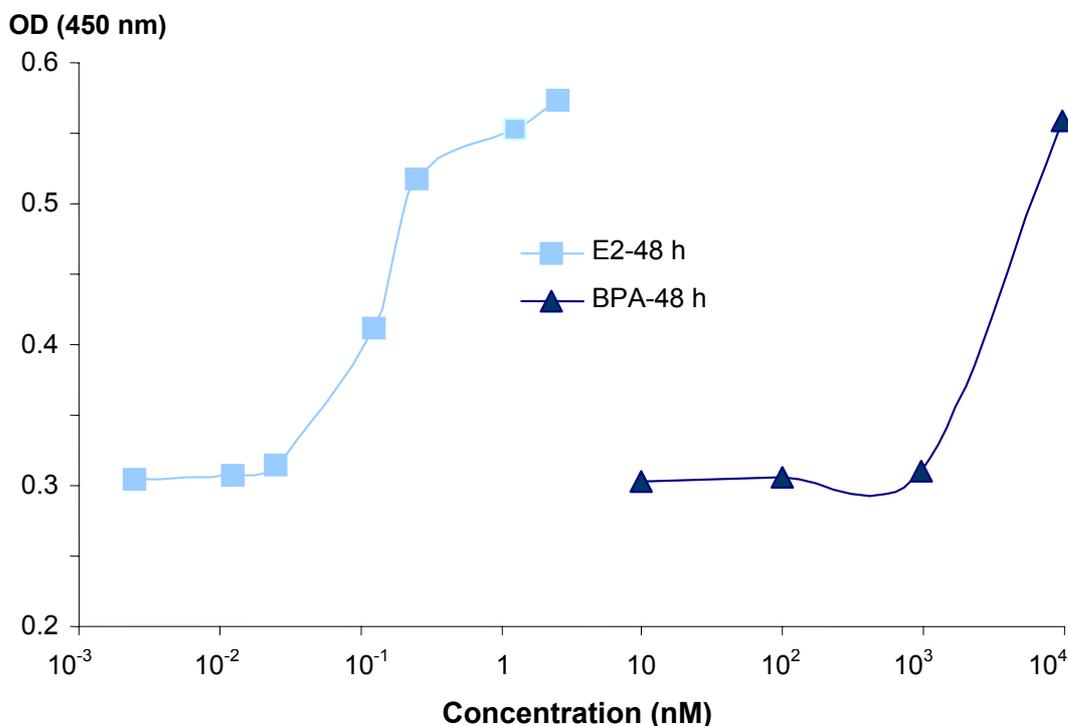


Fig. 5.19: The comparison of BPA response with these of 17β-Estradiol in optimized yeast assay.

5.6.2. Effect of the pre-treatment of H4IIE cells with 2,3,7,8-TCDD on the P450 induction

P450s are major oxidative enzymes that metabolize xenobiotics and the modulation of these enzymes can markedly affect toxicity and carcinogenicity [159]. The changes in P450 enzymes caused by BPA *in vivo* were also reported [160]. Although the biological effects of BPA such as carcinogenicity have been described [161], little is known about the ability to modulate P450 enzyme activities. In this part, we have investigated the ability of BPA to induce CYP1A enzyme in H4IIE cells and the effect of a pre-incubation of the cell line with the good known potent inducer TCDD overall activity expression in the system.

Figure 5.20 present the EROD activity of BPA in EROD assay and in combination with TCDD (pM) for 24 h, it could be seen that BPA alone affect slightly the induction of CYP1A1 enzyme in terms of EROD activity, but the pre-treatment of the H4IIE rat cells increase greatly the activity of this enzyme in the whole of the system.

In conclusion, Bisphenol A is unable to induce CYPA1A but the pre-treatment of H4IIE cells with TCDD enhance and stabilize the induction of this enzyme.

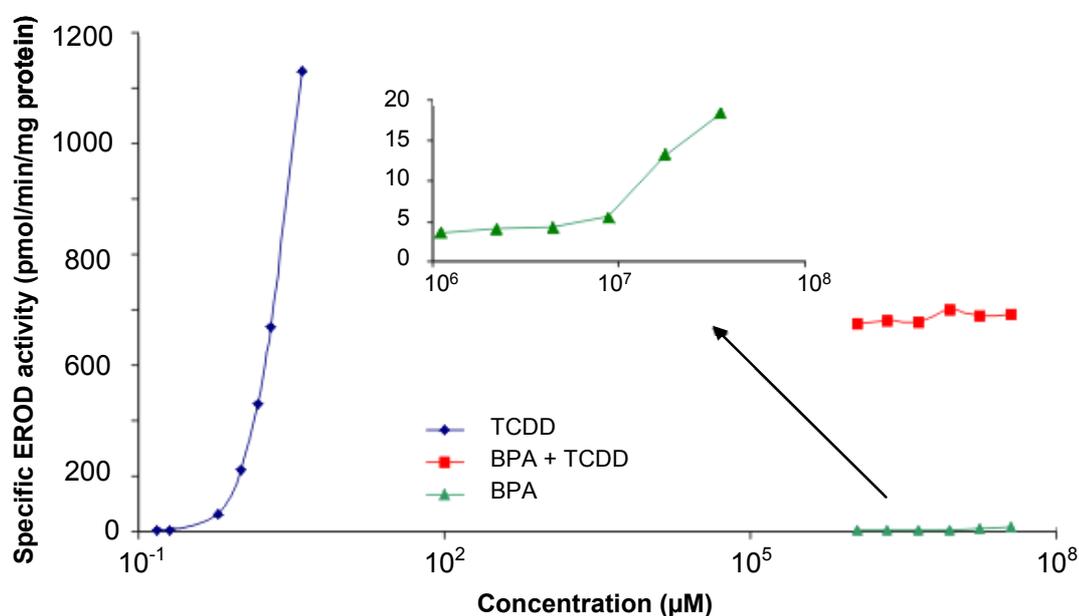


Fig. 5.20: Effect of the pre-incubation of H4IIE cells with TCDD on EROD-activity in presence of Bisphenol A.

5.6.3. Determination of cytotoxicity after incubation of cells with Bisphenol A using rezurufin assay

For determination of cytotoxicity of BPA during the metabolic activation step, H4IIE cells were incubated with BPA alone, and in combination with TCDD for 48 h. The results are plotted in Fig. 5.20; the percentage of intact cells is plotted as function of the concentration of BPA.

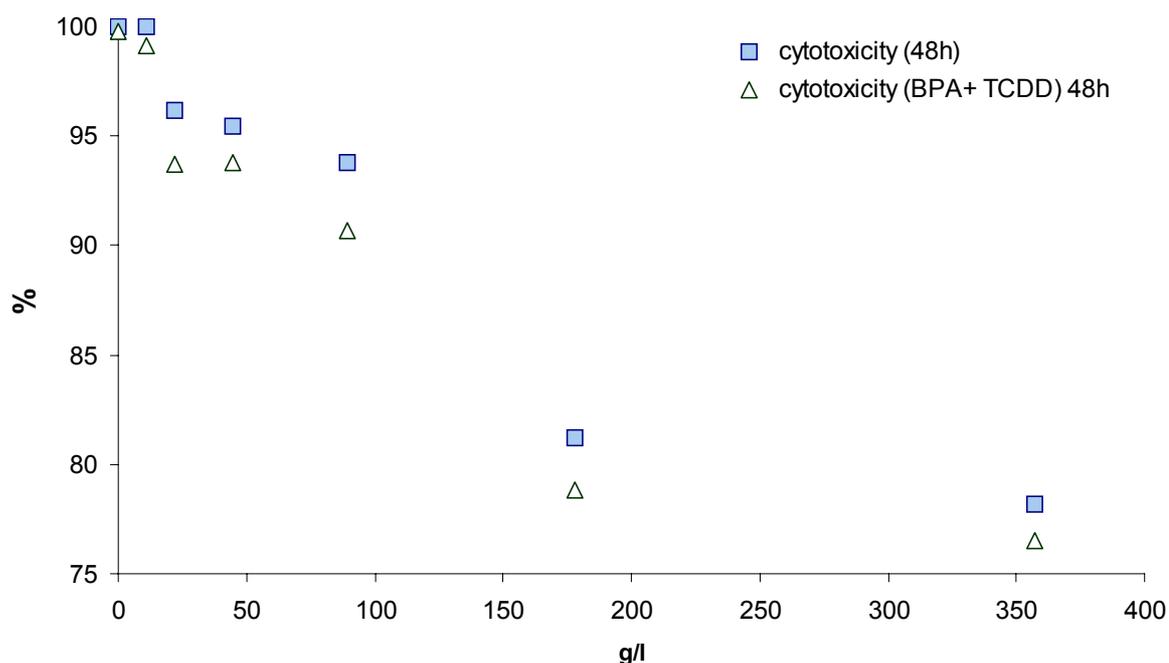


Fig. 5.20: Cytotoxicity of BPA concentration on the H4IIE hepatoma cells.

It was reported that after 48 h of incubation of H4IIE with concentration of BPA ranging from 11 to 357 g/l, the cytotoxicity is registered for high concentrations of BPA, up to 100 g/l, and less than 80 % of the cells are still living. Additionally, the cytotoxicity of BPA increases slightly in the case of TCDD pre-treated cells. Based on these results, non-cytotoxic concentrations were used in the metabolism, less than 25g/L.

5.6.4. Assay Optimization of metabolites extraction

After incubation of BPA with H4IIE cells, the metabolites should be extracted, which requires a good choice of suitable extraction solvent or extraction method. For this

reason three solvents were tested and compared for their of extraction recovery (Fig. 5.21).

The diethyl ether and acetone extraction gave unstable yield (between 20 and 110%) because of rapid evaporation of these solvents; furthermore laborious and hard-to-standardize method (easy evaporation of DEE, denaturated protein layer for DEE extraction, high content of water for acetone/ Na_2SO_4 extraction).

The SPE extraction, offer a stable extraction recovery of BPA about 70%. From three experiments, though probably it is the best possibility for extraction of metabolites.

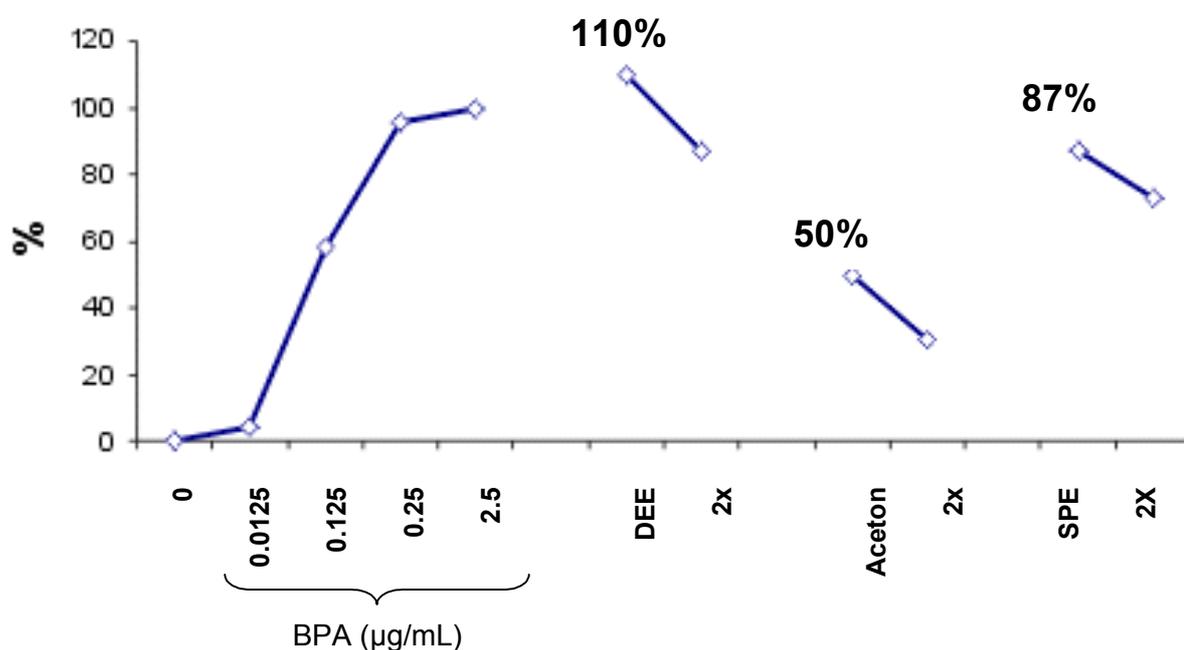


Fig. 5.21: Extraction recovery for different solvents used (2x mean 2 times dilution)

5.6.5. Modulation of Estrogenic activity of BPA after metabolic activation by H4IIE cells

After Incubation of TCDD-pre-treated H4IIE cells with BPA (2.5 mg/L) for 24h, the metabolites were extracted using HLB method and analyzed using yeast assay. The estrogenic activity of BPA was significantly reduced (Fig 5.22) to 10 %. After deglucuronidation of the metabolites, the estrogenic activity is increased; the gluco-conjugated metabolic fraction suggests the presence of conjugated metabolites.

Thus mean, that H4IIE cells converted BPA to conjugates, this kind of metabolites are known to be devoid of estrogenic activity [162].

Additionally, it can be concluded from Fig. 5.22, that after extraction the estrogenic activity of not metabolized BPA decreased, it is due to the losses during the extraction step, we are not sure then whether all the metabolites of BPA are extracted and tested in yeast assay. For this reason an investigation to avoid an extraction step was carried out.

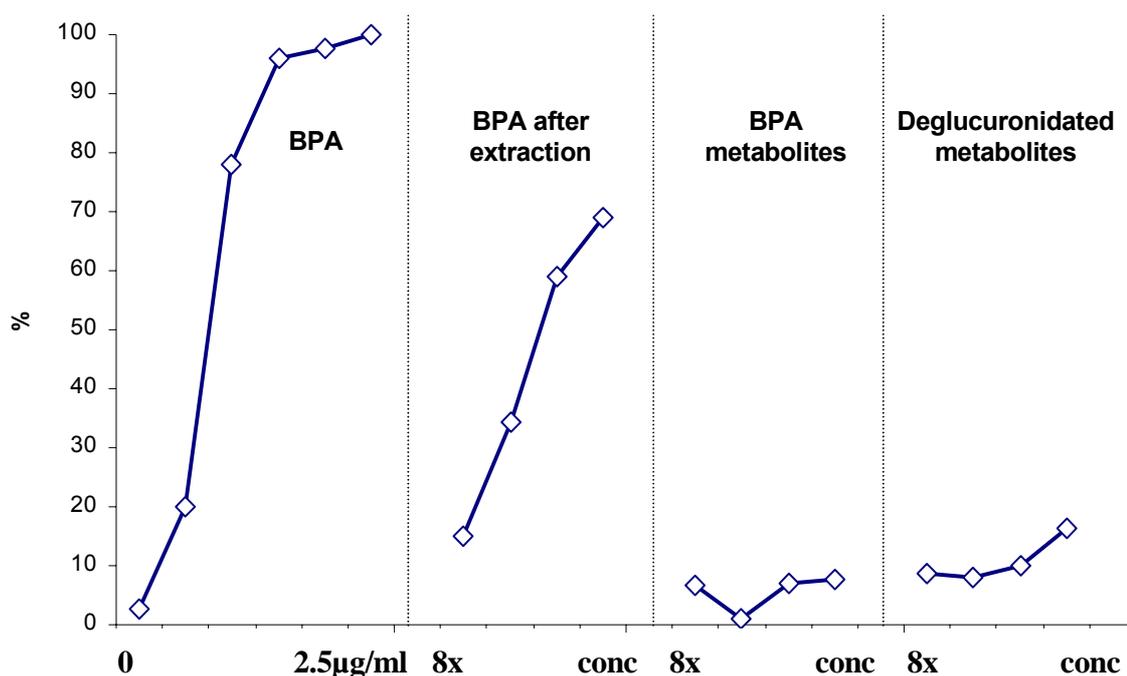


Fig 5.22: Modulation of estrogenic activity of BPA after metabolic activation (8x mean 8 times dilution)

5.6.6. Assessment of metabolic activation protocol without extraction

A metabolic experimental protocol without extraction step was developed. After metabolic activation, BPA and metabolites dissolved in DMEM culture medium were directly transferred to the yeast assay culture. The ratio of the mixture (DMEM: yeast culture media) was optimized, the 2:1 ratio was finally used because it allows a normal growth and activity of yeast cells as in normal growth media.

The results obtained using this modified protocol is reported in Fig. 5.23. As can be seen, the estrogenic activity of BPA is decreased after metabolism, consistent with

the previous finding using the extraction. The modified protocol present a great advantage, because the estrogenic activity measured by Yeast assay present the activity of the whole ensemble system after metabolism (BPA + metabolites), and it is more reliable and realistic.

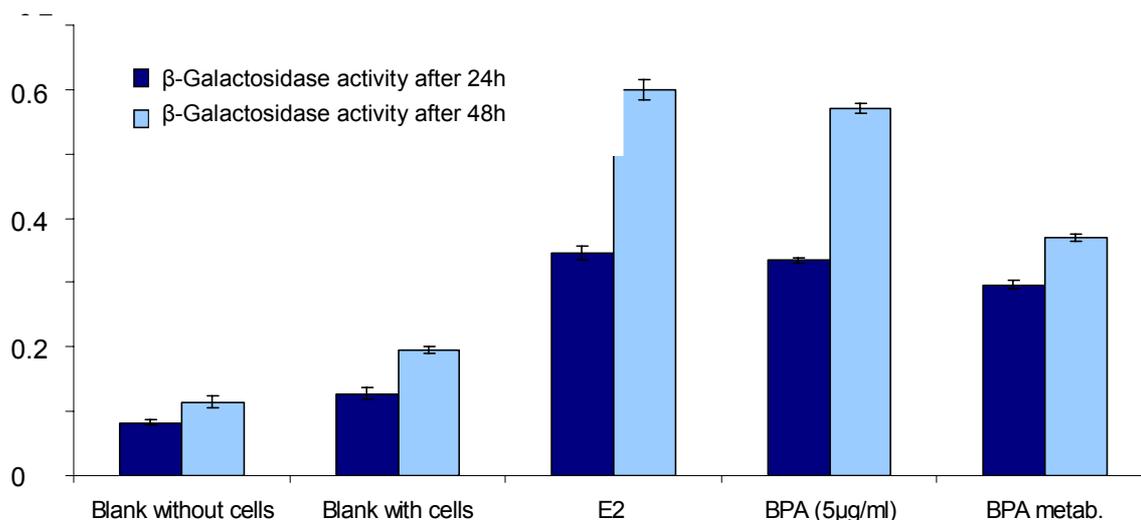


Fig 5.23: Modulation of estrogenic activity of BPA after metabolic activation using a modified protocol without extraction

5.6.7. Miniaturization of the metabolic activation protocol on 96 well plate

The miniaturization of the metabolic protocol present many advantages:

- easy to manage protocol, the same plate used in the metabolism step, will be used for the second step of the protocol in yeast assay
- no transfer or extraction of BPA or metabolites, avoiding any risk of losses
- low cost, small amount of media and used chemical, no use of extraction solvent
- a parallel quantification of Enzyme activity by performing the EROD bioassay, allowing the control of the CYP1A1 expression and by the way the system performance

The modulation of estrogenic activity of Bisphenol A using the developed micro-bioactivation protocol was performed; the TCDD-pre-treated H4IIE hepatoma cells were incubated in 96 well plates with 5 mg/L of BPA. At different incubation time the estrogenic and CYP1A1 enzyme activity were tested in parallel. The results are presented in Fig. 5.24.

During the metabolic activation both of the EROD activity of the system and Estrogenic activity of BPA are decreasing. That means that the P450 enzyme expressions control the metabolism of BPA during the bioactivation process. The used metabolic approach presents the first attempt to regulate and to control the enzyme induction during drug metabolism.

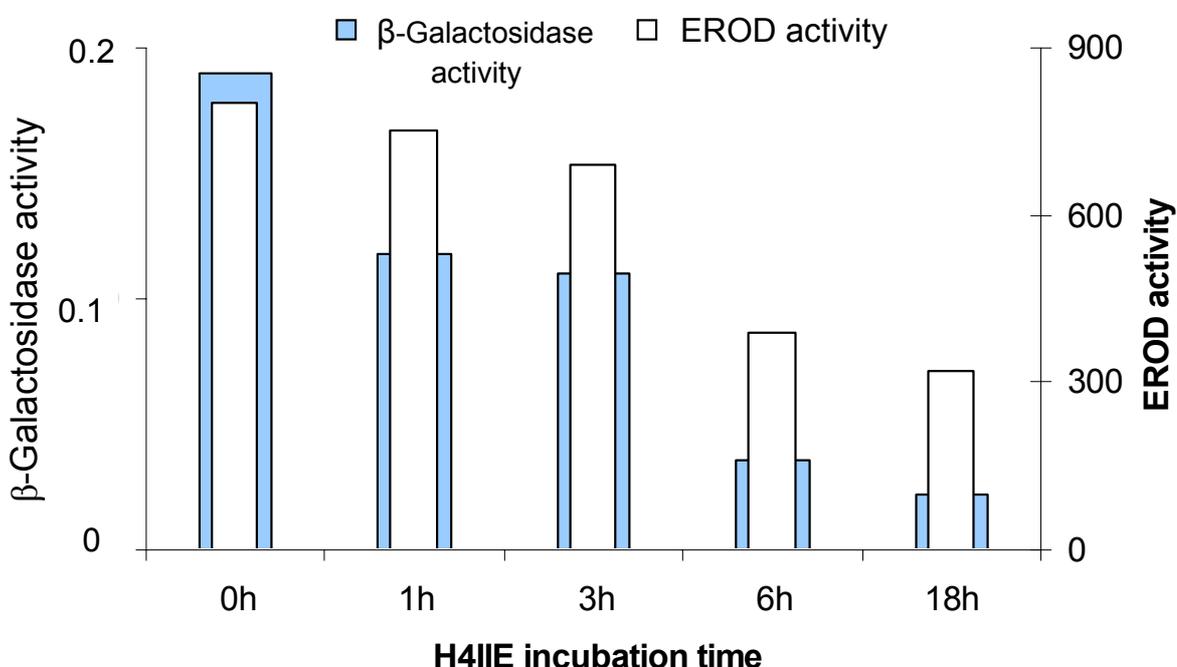


Fig 5.24: Modulation of Estrogenic- and EROD- activity of BPA system during the metabolism, using micro-bioactivation protocol

5.6.8. Investigations of Bisphenol A metabolism

The results obtained by the present study are consistent with the previous investigations concerning metabolic activation of BPA using rat system.

Most studies regarding the role of metabolism and metabolites in estrogenicity have been performed with rodents. *In vitro* studies with rat hepatocytes and *in vivo* studies with rats have shown that the major metabolite of bisphenol A (BPA) is a glucuronide.

This phase II metabolite is predominantly formed in the liver and excreted in the bile [163-165]. In addition, at least four metabolites, among others a monosulfate and 3-OH BPA were also formed in the rat, although quantitatively less important [164].

Experiments with different isoforms of UGT showed that UGT2B1 is probably the most important glucuronidation enzyme in the rat [166].

In fish, the glucuronidation of BPA has also been reported to occur easily. This was illustrated by the presence of BPA glucuronide in the bile of caged fish that were exposed to sewage effluent [167].

Toxicokinetic studies with rainbow trout showed that the formation of BPA glucuronide can reach plasma concentrations, which are about twice that of the parent compound [168]. Thus, it can be concluded that in both mammalian and piscine systems the formation of glucuronides is the preferred metabolic pathway. This is to be expected as BPA and several of its analogs contain (several) hydroxy groups, which are highly susceptible to phase II metabolism.

Glucuronidation of BPA should be considered as detoxification process, as several investigators showed that these metabolites lacked or had decreased binding affinity for the ER α or ER β in mammalian systems [165, 169]. At present, there is no indication that these glucuronides would behave differently in piscine or avian systems.

However, in the rat certain minor metabolites of BPA do possess estrogenic activity, in some cases exceeding that of the parent compound. The biological relevance of these estrogenic metabolites is unknown.

Limited information is available about the biodegradation of BPA in the environment. Bacteria from sewage sludge were able to degrade BPA to an intermediary metabolite 4,4'-dihydroxy- α -methylstilbene, that has a structural resemblance to diethylstilbestrol (DES). However, this compound is easily further degraded to 4-hydroxybenzaldehyde and 4-hydroxyacetophenone [170, 171]. The ecotoxicological significance of this formation process is presently unknown, but in general, BPA is considered as a readily biodegradable compound according to OECD standards [172].

6. Conclusion

Bio-analysis of dioxin-like compounds is an important tool for a comprehensive analysis of environmental matrices, foodstuffs, and substrates of technical origin. In addition, the molecular mechanism for dioxin-like compounds working in dioxin bioassays allows the comparison with data generated by chemical analysis. Uncertainties of both methodological approaches and their comparison were elaborated. In consequence, an inter-laboratory study on PCDD/F determining bioassays was performed, that presents for us the first exercise to validate the micro-EROD bioassay in an international manner.

Generally, for the bioassays investigated in this study, preliminary conclusions have been drawn by the critics of bio-analytical methods for the determination of PCDD/F, WHO-PCB and related compounds. The results obtained from the analysis of the biological matrixes (cod liver and salmon samples) showed that micro-bioassay is able to predict the WHO-TEQ level and correlate well with the chemical analysis using GC/MS. However, for fly ash matrix, containing elevated amounts of carbon, the results were lower than those from chemical analysis, because of the low efficiency of the used extraction procedure.

On the other hand, in order to reduce the risk for false negatives, the TEQ contribution in the partial AhR antagonists like mono- and diortho PCB require further addition. It should however be noted that non-additive interactions is a limitation in the WHO-TEF approach and not in the bioassay approach, which always reflects the combined effect of all AhR-interacting compounds in a sample. Additionally, in the present thesis, micro-EROD bioassay was investigated for screening dioxin-like activity in suspected leachate-polluted groundwater; the investigated samples present a weak EROD activity, due to the presence of non-persistent compounds. As samples can be complex and some contaminants might interfere with the EROD activity induced by other contaminants, the concern about false negatives must be kept in mind. Indeed, several factors, such as adsorption of dioxin-like compounds to particulate matter or interference with enzyme induction or activity by volatile organic chemicals, can diminish the EROD response. Thus, application of the P450 Enzyme induction in EROD-assay for testing groundwater samples can be regarded as an early warning tool to initiate a more detailed cause-analysis and to guide subsequent

chemical identification.

Furthermore, the use of H4IIE rat hepatoma cell line was also employed as a cell model to screen 7-ethoxyresorufin O-deethylase (EROD)-TCDD equivalents (EROD-TEQ) of human breast milk samples collected from Denmark and Finland. The EROD-TEQ was correlated well with TEQ generated by chemical analysis, but still lower for the both cases. Furthermore, this difference was higher in case of the Finnish samples than for those from Denmark, suggesting the presence of other compounds, which have a slight antagonistic interaction with the Ah-receptor.

For further improvement of the EROD-TEQ method, a more practical consideration of the complex interactions of the AhR agonists and antagonists present in sample mixtures should be determined. Nevertheless, our results suggest, that the EROD-TEQ assay was a sensitive screening tool, and is therefore particularly valuable to a cost conscious, high-through put breast milk-monitoring program.

Finally, other aspect of the use of an expression of P450 in H4IIE was reported. The ability of some natural halogenated organic compounds to bind to AhR receptor was performed with micro-EROD bioassay. From the physico-chemical parameters determination of Q1 and TBA, reported by Vetter et al 2004, it was clear that the pyrrole units of Q1 cannot occupy planar conformation. Consistent with that, EROD induction potency, which typically is a function of planarity of TCDD-like compounds, was low which is in agreement with the conclusion that Q1 is not a planar potent molecule.

The forthcoming screening thousands of chemicals, together with the increasing widespread use of many different *in vitro* assays for different endocrine activities, no single assay can be expected to be “the best” to assess estrogenicity, and any response in the *in vitro* assay needs to be confirmed by *in vivo*. Only by using a suite of assays in this way, it will be possible to minimize the chances of wrong-labeled chemicals as endocrine disruptors.

Chemicals may be converted to active or inactive estrogens following *in vivo* metabolism. In order to provide an even realistic screening tool, a biotransformation step was included in the Recombinant yeast assay. Bisphenol A was chosen as model compound because of the widely number of previously investigations that reported different results about its estrogenicity. In our study, we have developed a two-stage approach coupling H4IIE cells incubation and recombinant yeast assay to assess the role of metabolism in modulating the estrogenic activity of BPA.

The H4IIE cells system retains various drug metabolizing enzymes and cofactors associated with phase I and Phase II, and is a useful system to study the intracellular target sites and temporal sequences leading to cell damage caused by chemicals and their metabolites. On the other hand, H4IIE cells are very sensitive and the P450 enzyme can be easily expressed and regulated by addition of Ah agonist compound, from example 2,3,7,8-TCDD.

Pre-incubation of bisphenol A with TCDD-induced H4IIE hepatoma cells resulted in lower estrogenic activity of the metabolites in the recombinant yeast assay than the parent compound. Accordingly, other studies have shown that Bisphenol A is metabolized *in vivo* or using primary rat hepatocytes cells to produce less estrogenic glucuronided metabolites. The pre-incubation of H4IIE cells with TCDD, allow the regulation of the expression of CYP1A1 enzyme. That could be easily quantified by micro-EROD assay; this concept has as an advantage, the control of the bioactivation step, and could also provide a simple addition to a sensitive *in vitro* screen bioassay, such as recombinant yeast assay, to provide more insight in the *in vivo* estrogenic potency of a suspected xenoestrogen.

7 Outlook

The induction of P450 enzyme in H4IIE cells presents a useful tool in ecotoxicological studies and should be more investigated for developing *in vitro* assays that could contribute in minimizing and avoiding animal's assays.

The developed bioactivation protocol for screening estrogenic activity of Bisphenol A after metabolism using a combination of H4IIE hepatoma and yeast cells is a reliable and promoting approach; however, more investigations still be needed for determination of metabolites generated by such system using analytical methods. Finally, the extrapolation of the test system for screening different environmental- and human samples should be investigated, to define the limits and performances of the developed approach in a practical manner.

8. Appendix

8.1. Dioxin laboratory

Equipment	Company	Type
Rotary evaporator	Büchi	EL 131
precision Balance	Sartorius	LC 4800 P
N ₂ -Thermoblock	Labour Technik Barkley	
Ultrasonic bath	Bandelin, Sonorex	RK 510 H
Drying oven	Heraeus-Wärmetechnik	WU 610
Laboratory machine for rinsing	Miele, Typ Mielabor	G 7733
Glassware	Volume	Trimble
Round flask	100, 250, 500, 1000 mL	NS 29/32
Erlenmeyer flask	50, 100, 250, 500, 1000 mL	NS 29/32
Soxhlet apparatus	500, 1000 mL	NS 29/32
Dropping funnel	100, 300 mL	NS 29/32
Graduated cylinder	25, 50, 100, 250, 500 mL	
Chromatographic column		
Pasteur pipette		
Solvents, adsorbents, consumptions	Package Quantity	Supplier
Acetone	4 L	Promochem
Acetonitrile	2. 5 L	Riedel-de-Haen
Benzene	2. 5 L	Promochem
Dichloromethane	4 L	Promochem
Chloroform	2. 5 L	Merck
Nonan	2. 5 L	Promochem
n- Hexane	2. 5 L	Promochem
Tetradecan	500 mL	Aldrich Chemical
Toluene	2. 5 L	Riedel-de-Haen
Aluminium oxide B Super I	500 g	ICN Biomedicals
Bio-Bed SX-8		Bio Rad
C-18	100 g	Bulb
Sodium chloride	5 Kg	Merck
Silica gel	1 Kg	Promochem
Florisil	500 g	Promochem
Glass wool	1 Kg	Neolab
Vials		neolab

8.2. Hepatoma cells culture zone

Instrument	Type
Autoclave	Varioklav 50050; H+P Labortechnik
Incubator	B 5061 EC/CO ₂ , BB 6060; Kendro
Luminescence measuring instrument	Lumicount; Packard
Fluorescence measuring instrument	Fluorspektra; Kendro
Pipettes	Gilson, Pipetman P; Abimed
Waters Bath	Köttermann; Ing.-büro Braun
Laminar flow	HeraSafe
Centrifuge	Biofuge 22R, Kendro
Centrifuge rotor	3747, 3743
Vortexer	Axiovert 25; Bender + Hobein
Pipettes	Eppendorf
Plates shaker	Titmax 100; Heidolph
Light-optical microscope	Zeiss

8.3. Yeast cells culture zone

Instrument	Type
Autoclave	Webeko; Schembe
Millipore water plant	Super Q; Millipore
Ultrasonification bath	Branson
Laminar flow	Kendro
Vortexer	VF2; Ika-Labortechnik
Magnetic stirrer	Ikamag; Ika-Labortechnik
Plates Photometer	SLT-Spectra; SLT-Lab instruments
Kuvettes Photometer	Unicam 5675; Unicam Limited
Vortexer	VF2; Ika-Labortechnik
Shaker incubator	Infors

8.3.1. Consumables

Material

Single-serving syringes
Reaction vessels Standard 1.5 mL
Cell culture Flasks 25, 75 cm²
Centrifugal tube 14 mL
96-well-Plates, Ft, transparent
Centrifugal tube 50 mL
Vials 1.2 mL with screw cap
Silica gel 60 (70-230 mesh), p.a.
Florisil (60-100 mesh), p.a.
Aluminiumoxid B Super1

Supplier

Becton Dickinson
Eppendorf
Nunc
Becton Dickinson
Greiner
Falcon
neolab
Promochem
Promochem
ICN

8.3.2. Chemicals

All the chemicals, unless stated otherwise, were of the highest available degree of purity, and did not undergo any further purification. The used water was filtered by a Millipore water treatment plant.

Reagent	Manufacturers	Location
Agar	Gibco-BRL	Freiburg
Agarose	Gibco-BRL	Freiburg
Ammonium persulfate	Fluka	Neu-Ulm
Ampicillin	Amersham-Buchler Inc	Braunschweig
L-Arginine Hydrochloride	ICN	Eschwege
BSA	Uptima	Montlucon Cedex (F)
Dicoumarin	Boehringer	Mannheim
Dimethylsulfoxide, DMSO	Fluka	Neu-Ulm
Ethinyl estradiol	Sigma	Deisenhofen
7-Ethoxyresorufin	Merck	Darmstadt
fetal calf serum (FCS)	Biochrom	Berlin
charcoal stripped FCS	Biochrom	Berlin
Glutaraldehyde solution, 1,2 %	Fluka	Neu-Ulm
Copper(II) sulfate -Pentahydrat	Merck	Darmstadt
Silica gel 60	Merck	Darmstadt
Potassium chloride	Fluka	Neu-Ulm
Potassium phosphate, dibasic	Fluka	Neu-Ulm
Nitrophenyl- β - Dgalactopyranoside	Merck	Darmstadt
PBS	Biochrom	Berlin
Penicillin/Streptomycin	Biochrom	Berlin
Resazurin	Sigma	Deisenhofen
Trypsin	Biochrom	Berlin

8.3.3. Strains of bacteria/ cell lines

Strain / cell line	Literature / source
H4IIE	Reuber, 1961; Pitot, 1964
Yeast SU	Routledge, 1996

8.3.4. Kits

Kit	Manufacturers	Location
BCA-Micro Assay Kit	Uptima/KMF	Montlucon Cedex (F) /Siegburg
Luciferase Assay System	Promega	Madison (USA)

8.3.5. Culture media, solutions, and buffers

All mediums, solutions and buffers were prepared with Millipore water (18 M μ /cm) and depending on the reagent autoclaved or sterile filtrated.

Culture medium

Composition of used culture media

DMEM-medium	Hydraulic fluid <i>Biochrom</i>	
	Glucose	1.0 g/L
	N-Acetyl-L-Alanyl-L-Glutamine	2 mM
	FBS	10 %
	Penicillin/Streptomycin	1 %
Yeast minimal-medium	KH ₂ PO ₄	13.61 g
	(NH ₄) ₂ SO ₄	1.98 g
	KOH-pellets	4.2 g
	MgSO ₄	0.2 g
	Fe ₂ (SO ₄) ₃ -solution	1.0 mL
	L-Leucine	50 mg
	L-Histidine	50 mg
	Adenine	50 mg
	L-Arginine-HCL	20 mg
	L-Methionine	20 mg
	L-Tyrosine	30 mg
	L-Isoleucine	30 mg
	L-Lysine-HCL	30 mg
	L-Phenylalanine	25 mg
	L-Glutamic acid	100 mg
	L-Valine	150 mg
	L-Serine	375 mg
H ₂ O	ad 1 L	
Yeast medium for storing at -20° C	Glucose solution	5.0 mL
	L- Aspartic acid solution	1.25 mL
	Vitamin solution	0.5 mL
	L-Threonine solution	0.4 ml
	Cupric solution	125 µL
	Minimal medium	45 mL
Yeast medium for storing at -80° C	Glucose solution	10 ml
	L-Asparagine solution	2.5 mL
	Vitamin solution	1.0 mL
	L-Threonine solution	0.8 ml
	Copper (II) sulfate solution	250 µL

Yeast growth-medium	Glucose solution	5.0 mL
	L-Asparagine solution	1.25 mL
	Vitamin solution	0.5 mL
	L-Threonine solution	0.4 mL
	Copper (II) sulfate solution	125 µL

The culture mediums are prepared with addition of 1.5% agar.

Solutions and buffers

Composition of used solutions and buffers for the EROD-Bioassay

PBS	NaCl	8.0 g
	KCl	0.2 g
	Na ₂ HPO ₄ *2H ₂ O	1.44 g
	KH ₂ PO ₄	2.2 g
	H ₂ O	ad 1 L
7-Ethoxyresorufin-solution. 400 µM	7-Ethoxyresorufin	5 mg
	Methanol	51 mL
Dicumarol 1 mM	Dicumarol	16.5 mg
	NaOH-solution. 0.1 M	clear solution
	TRIS-buffer. 50 mM	48 mL
Resazurin-solution	Resazurin	100 mg
	PBS	10 mL

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