

Review Articles

Cell Bioassays for Detection of Aryl Hydrocarbon (AhR) and Estrogen Receptor (ER) Mediated Activity in Environmental Samples

Klara Hilscherova¹, Miroslav Machala², Kurunthachalam Kannan³, Alan L. Blankenship^{3,4} and John P. Giesy³

¹Department of Environmental Chemistry and Toxicology, Faculty of Science, Masaryk University, Veslarska 230B, 63700 Brno, Czech Republic

²Veterinary Research Institute of Veterinary Medicine, Hudcova 70, 62132 Brno, Czech Republic

³Department of Zoology; National Food Safety & Toxicology Center; Institute for Environmental Toxicology, Michigan State University, East Lansing, Michigan, USA, 48824

⁴ENTRIX Inc., East Lansing, MI, USA

Corresponding author: Klara Hilscherova; e-mail: klara@chemi.muni.cz

DOI: <http://dx.doi.org/10.1065/espr2000.02.017>

In vitro cell bioassays are useful techniques for the determination of receptor-mediated activities in environmental samples containing complex mixtures of contaminants. The cell bioassays determine contamination by pollutants that act through specific modes of action. This article presents strategies for the evaluation of aryl hydrocarbon receptor (AhR)- (hereafter referred as dioxin-like) or estrogen receptor (ER)-mediated activities of potential endocrine disrupting compounds (EDCs) in complex environmental mixtures. Extracts from various types of environmental or food matrices can be tested by this technique to evaluate their 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) or estrogenic equivalents (E₂-EQs) and to identify contaminated samples that need further investigation using resource-intensive instrumental analyses. Fractionation of sample extracts exhibiting significant activities, and subsequent reanalysis with the bioassays can identify important classes of contaminants that are responsible for the observed activity. Effect-directed chemical analysis is performed only for the active fractions to determine the responsible compounds. Mass-balance estimates of all major compounds contributing to the observed effects can be calculated to determine if all of the activity has been identified, and to assess the potential for interactions such as synergism or antagonism among contaminants present in the complex mixtures. The bioassay approach is an efficient (fast and cost effective) screening system to identify the samples of interest and to provide basic information for further analysis and risk evaluation.

Keywords: Complex mixtures; dioxin-like activity; endocrine disruptors; estrogen receptor-mediated activity; fractionation; *in vitro* cell bioassays; toxic equivalents

Introduction

There is increasing concern over the potential adverse effects of xenobiotics present in the environment and food-stuffs on human and wildlife populations. Two groups of toxicants of current interest are dioxin-like and (anti-)estrogenic chemicals. Many of these ubiquitous compounds are hydrophobic, lipophilic and resistant to biological and chemical degradation. These properties impart persistency and

propensity to bioaccumulate and biomagnify to concentrations that can cause deleterious effects on cells and tissues. In the environment, chemicals occur as complex mixtures including different congeners and isomers of both natural and anthropogenic origin. The concentrations and toxic potencies of compounds present in complex mixtures can range over several orders of magnitude. In addition, interactions among different classes of compounds (e.g., estrogenic vs. anti-estrogenic) can modulate the toxic potential. This complicates hazard evaluation and risk assessment of complex mixtures of xenobiotics. Furthermore, toxic effects of some contaminants, even those, which are analytically determined, are not well characterized. There are many potentially significant classes of contaminants that are not studied in detail, primarily due to a lack of suitable instrumental techniques or analytical standards. In other words, chemical analysis has been used to identify and quantify only those chemicals for which analytical techniques and standards are available. Instrumental analyses do not account for interactions among the chemicals in complex mixtures and provide little information on their biological effects. Chemical analyses can also be costly and time consuming. Thus, chemical analyses can underestimate the potential risks posed by these chemicals; some toxicologically important compounds could be overlooked.

In vitro cell bioassays offer a rapid, sensitive and relatively inexpensive solution to some of the limitations of instrumental analysis. They enable estimation of total biological activity of all compounds that act through the same mode of action present in extracts of any environmental media. Bioassays also integrate possible interactions among chemicals. In this review, the applicability of *in vitro* cell bioassays for assessment of two toxicological modes of action, dioxin-like toxicity and estrogen receptor-mediated activity, is evaluated. Several reviews concerning dioxin-like and estrogenic activities of xenobiotics have appeared recently [1-4]. In our paper, the strategy of the cell bioassay approach for evaluation of receptor mediated activity of complex mixtures is presented, including fractionation procedures, mass balance calculations, toxicant identification and risk assessment. Also,

the classes of AhR-agonists and compounds that have been shown to elicit endocrine disrupting potential are summarized.

In vitro methods, while a useful adjunct to instrumental analyses are not a substitute for in vivo toxicological studies. The in vitro assays discussed in this review are useful as sensitive integrating methods that provide quantitative estimates of the total activity of particular receptor-mediated responses. However, since they do not account for bioavailability and predict who-organism responses, the results of the in vitro bioassays provide information that is similar to that provided by instrumental analyses. Just as with information on the concentrations of residues obtained from instrumental analyses, the results of the bioassays need to be compared to appropriate toxicant reference doses, determined from in vivo studies.

1 Dioxin-Like Activity

Chemicals that elicit toxic effects similar to that of TCDD, known as dioxin-like chemicals, are of great concern due to their ability to cause hepatotoxicity, embryotoxicity, teratogenicity, immunotoxicity, dermal toxicity, lethality, carcinogenesis, wasting syndrome and tumor promotion in many different species at low concentrations [5,6]. A number of studies have demonstrated that several toxic and biochemical effects caused by dioxin-like chemicals are mediated through aryl hydrocarbon receptor (AhR) [7,8]. The AhR, which belongs to the basic helix-loop-helix protein family [9], is a ligand-dependent transcription factor located in the cytosol, complexed with heat shock proteins. It has been shown that the strength with which congeners bind to the AhR is directly proportional to the toxicity, enhanced gene transcription and enzyme activities mediated by the AhR mechanism [10]. The role of AhR in mediating toxic and biological effects of dioxin-like chemicals has been well documented in a number of studies, even though the exact biochemical mechanism leading to the wide spectrum of toxic responses is yet to be elucidated [11]. After binding of ligand to cytosolic AhR, heat shock proteins dissociate from the complex, the receptor ligand complex is activated and translocated to the nucleus, where it forms a dimer with the Ah receptor nuclear translocator (ARNT) protein and possibly other factors. The heteromeric ligand:AhR:ARNT complex binds with high affinity to specific DNA sequences, the dioxin-responsive element (DRE). The binding to the DRE results in DNA bending, disruption of chromatin and nucleosome and thus increased promoter accessibility and transcriptional activation of adjacent responsive genes (\rightarrow Fig. 1) [11,12].

The traditional, well-known ligands for AhR have been described as hydrophobic aromatic compounds with planar structure of a particular size of the molecule or a part of the molecule, which fits the binding sites [13,14]. Thus, the ability of these ligands to bind to the AhR and to cause toxic effects greatly depends on their structure and substitution pattern. These include planar congeners of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs and PCDFs), chlorinated azobenzenes and azoxybenzenes, polychlorinated biphenyls (PCBs), several polycyclic aromatic hydrocarbons (PAHs) and polychlorinated naphthalenes (PCNs) [15]. Other chemicals suggested as potential AhR agonists due to their stereochemical configuration, but not yet experimentally confirmed, in-

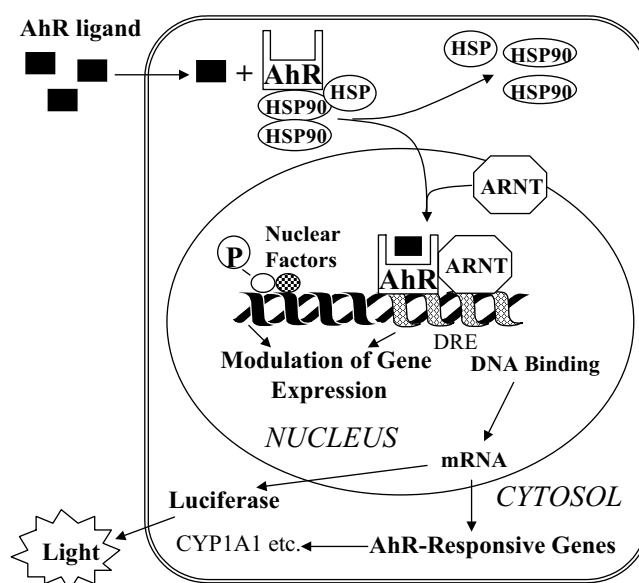


Fig. 1: Mechanism of aryl hydrocarbon receptor (AhR)-mediated response in cell bioassay (adapted from [15]). For description see text. HSP = heat shock proteins, P = phosphates: phosphorylation is an important regulatory factor for receptor function

clude polybrominated and chloro-/bromo- analogs of the previously listed classes of compounds [16], alkylated-chlorinated dioxins and furans, chlorinated dibenzothiophenes, chlorinated xanthenes and xanthenes [17], polychlorinated diphenyltoluenes (PCDTs), anisols (PCAs), anthracenes (PCAN), fluorenes (PCFL) and others [18]. Recently new types of relatively weak AhR ligands or inducers (compared to TCDD) have been identified, which include both natural and synthetic compounds [11]. These compounds deviate from the traditional criteria of planarity, aromaticity and hydrophobicity. The natural compounds that bind to the AhR include, among others, indoles, tryptophan-derived products, oxidized carotenoids and heterocyclic amines. Some pesticides or drugs with various structures, such as imidazols and pyridines also possess the AhR binding ability. These ligands act as transient inducers and bind to the AhR with weak affinity and are rapidly degraded by the induced detoxification enzymes.

2 Estrogenic Activity

In recent years there has been increasing interest in chemicals that can modulate the endocrine system. Such compounds have the potential to disrupt normal reproduction or developmental processes which can lead to adverse health effects such as compromised reproductive capacity, breast and testicular cancer, reproductive dysfunction such as feminization or demasculinization of males and other adverse effects. A wide range of compounds including natural products, pharmaceuticals and industrial chemicals has been shown to be estrogen mimics. As observed in recent studies [19], some hormone-mimicking chemicals can elicit multiple endocrine disrupting activities that are mediated by various mechanisms of action, some of them may be active only during certain stages of development. Their effects can be mediated through receptor-mediated mechanisms (such as

estrogen or androgen receptor), but some compounds can disrupt hormone functions at different levels of the endocrine system, not directly interacting with the receptor. *Estrogen-like* compounds exert effects by resembling those of estrogen but not mediated by the estrogen receptor (ER) [2]. Various modes of actions have been reported, which include binding of chemicals to other nuclear receptors, which then interact with an estrogen responsive element; acting through other receptors and/or signal transduction pathways; modulations of steroidogenesis and catabolism of active steroid hormones [20]. *Estrogenic* compounds are characterized by their ability to bind to and activate the estrogen receptor, which is a transcription factor belonging to the steroid receptor family. While there are structural similarities among some compounds that are ER agonists, other ER-active compounds do not share similar structures. Upon binding of an estrogenic compound to the ligand binding domain of the ER (located predominantly in the nucleus), the associated heat shock protein complex, which masks the DNA binding domain, dissociates and subsequently the ligand occupied receptor dimerizes. The homodimer complex interacts with specific DNA sequences referred to as estrogen response elements (EREs) located in the regulatory regions of estrogen-inducible genes. ER complexes bound to an ERE recruit additional transcription factors, leading to increased gene transcription and synthesis of proteins required for expression of hormonal action (\rightarrow Fig. 2) [21,22]. A series of natural and synthetic endocrine disrupting compounds have been identified by different *in vivo* and/or *in vitro* methods. Numerous specific testing systems have been developed for the detection of effects at different levels of the endocrine system [1,3]. Examples of xenoestrogenic compounds including natural and major classes of industrial contaminants are presented along with the method used to determine their relative estrogenic potency (\rightarrow Table 1).

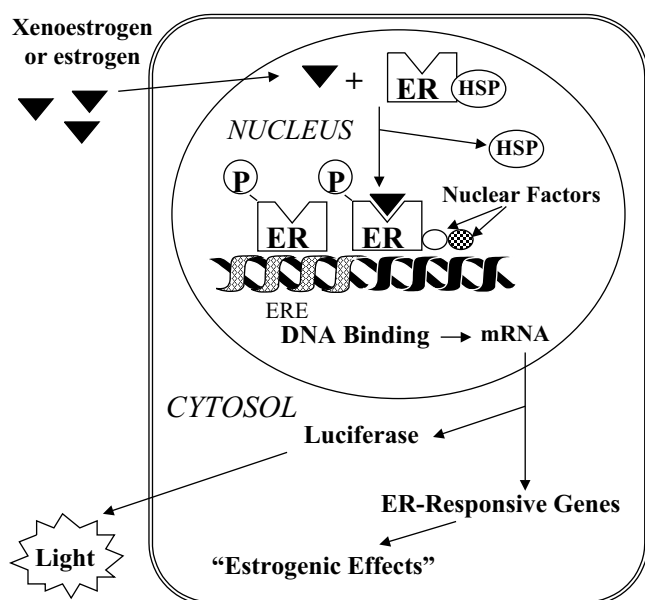


Fig. 2: Mechanism of estrogen receptor (ER)-mediated response in cell bioassay (adopted from [23]). For description see text. HSP = heat shock proteins, P = phosphates: phosphorylation is an important regulatory factor for receptor function

3 Cell Bioassay Approaches

Bioassays based on the responses of either wild type or genetically engineered eukaryotic cells enable the assessment of potencies of individual chemicals or complex mixtures of environmental contaminants in extracts to cause AhR- or ER-mediated effects. Either endogenous responses or exogenous reporter systems are incorporated into the cell are used for the measurements. The induction of transcription by the responsive genes following the exposure of cells to specific ligands or mixtures of compounds can be assessed by measuring endogenous or genetically engineered responses such as protein expression by measuring the amount of protein directly or by measuring an enzyme activity.

The endogenous responses for AhR binding, increased expression and induced activity of cytochrome P4501A1 and its monooxygenase activities, such as 7-ethoxyresorufin O-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) are measured as markers of responses to AhR agonists [65,66]. The ER-mediated activity can be examined by the determination of specific gene products such as vitellogenin, pS₂ or steroid hormone binding globulins [23,67,40]. List of some animal and human cell lines used for the detection of *in vitro* TCDD-like or estrogenic activity is shown (\rightarrow Table 2, p. 164).

Recombinant cell lines are prepared by transient or stable transfection of wild type cells with reporter genes under transcriptional control of either DRE or ERE. *Transient transfection* is relatively fast and easy to perform, but variations in transfection efficiency warrant the need for co-transfection with internal constitutive control to correct results for the transfection efficiency. It can be used only for short-term studies, because transgenes are usually lost after about 72 hrs. In addition, physiological conditions including target DNA accessibility or overexpression of receptors or target DNA do not reflect the normal cell function [21]. *Stable transfection* requires cotransfection of the plasmid with the gene of interest and plasmid encoding the marker for drug resistance, enabling selection of only successfully transfected cells and their development into a stable cell line. The gene of interest becomes a permanent part of the cell genome. These cell lines are suitable for longer-term experiments and their results are more reproducible. Transfection with recombinant expression vectors, which contain selected responsive elements upstream of a reporter gene produces a cell bioassay for specific class of chemicals. The most common reporter genes are firefly luciferase (luc), alkaline phosphatase (PAP), chloramphenicol acetyl transferase (CAT), or β -galactosidase (LacZ) [21,68]. Either native AhR or ER is used or the cells can be transfected with chimeric receptor linked to the recombinant reporter gene. Introduction of a complete receptor-reporter system into the cells enables the development of responsive bioassays from cells with no endogenous receptor present, such as in yeast cells [42]. Recombinant yeast cells are easy to develop and maintain and they are free of steroid receptor background, which causes potential interference in the assay. But some differences in ligand specificities exist between animal cells and yeast, also the ability of yeast to metabolize proestrogens to estrogens have been reported [23].

Table 1: Examples of endocrine disrupting compounds: natural products (phytoestrogens and mycoestrogens) and synthetic chemicals

Compound	Mode of action	Assay	Reference
1. PHYTOESTROGENS			
Indole-3-carbinol	ER agonist	RER (MCF-7-luc), YES	23, 24
β -Sitosterol	ER agonist, androgenic after metabolization	YES, in vivo fish	24, 25
Coumestrol	ER agonist	RER (MCF-7-luc), YES in vitro – ER mediated alkaline phosph. induction	23, 24 26
Enterolactone, Enterodiol	decreased aromatase enzyme activity	in vitro – human cell culture system	27
Genistein	ER agonist estrogenic	RER (ER-CALUX) in vitro and in vivo vitellogenin production	28, 26 29
Biochanin A, Daidzein, Equol	ER agonists, estrogenic	in vitro and in vivo vitellogenin production in vitro – ER mediated alkaline phosph.induction	29 26
Quercetin, Naringenin, Luteolin	Estrogenic, antiestrogenic	CB-ER, RER	30, 31
Apigenin, Chrysin, Kaempferol, Hydroxy- and Methoxy-flavons	ER agonists	RER (MVLN)	32
2. MYCOESTROGENS			
Zearalenone	ER agonist	CB-ER, RER, Vtg – in vitro	30, 33
3. PHARMACEUTICALS			
Flutamide	antiandrogenic activity	YES	19
Tamoxifen	antiestrogenic drug binding to ER, ER antagonist or agonist	in vitro cell line tests, in vivo E-screen and other effects	34, 35, 36 37
Hydroxytamoxifen	antiestrogenic and antiandrogenic activity	YES, E-screen and other effects	19, 37
Nafoxidine, Clomiphene	ER agonist	YES	24
Ethinylestradiol	ER agonist	in vitro, in vivo	29, 25
4. ADDITIVES			
Parabens	ER agonists	CB-ER, YES, in vivo uterotrophic response	38
t-Butylhydroxyanisol	estrogenic	E-screen	39
5. PESTICIDES			
5.1 Insecticides			
o,p'-DDT	ER agonist, antiandrogenic activity	YES, RER (ER-CALUX), Vtg – in vitro	24, 28, 19, 40
o,p'-DDD, o,p'-DDE	ER agonists	YES	24
p,p'-DDE	androgen receptor antagonist, weak ER and androgen receptor agonist antiandrogenic and weak antiestrogenic activity	CB-androgen receptor, in vivo mice study YES	41 19
p,p'-DDD	ER agonist	YES, CB-ER, RER (MCF-7-luc)	42
p,p'-DDT	ER agonist, estrogenic	E-screen	29
Kepone	ER agonist, estrogenic - after metabolization	RER (ER-CALUX), E-screen, in vitro + in vivo	28, 29, 36
Endosulfan, Dieldrin, Lindane	ER agonist	RER (ER-CALUX)	28
Toxaphene	estrogenic	E-screen	43
Methyl parathion	estrogenic	YES, Vtg – in vitro in vivo effects on estrus cycle in mice	44 45
Chlordecone	estrogenic	YES, Vtg – in vitro	44
Chlordane	ER agonist	RER (ER-CALUX) in vivo effects on endocrine function in mice	28 46
Methoxychlor	ER agonist - after metabolization	RER (ER-CALUX) in vitro + in vivo	28 36
Carbamate insecticides (Aldicarb, Bendiocarb, Carbaryl, Methomyl, Oxamyl)	endocrine modulators, non-ligand binding	in vitro modulation of estrogen and progesterone receptor in human breast and endometrial cancer cells	42
Pyrethroid insecticides (Sumithrin, Fenvalerate, Allethrin, Permethrin)	estrogenic (different mechanisms)	in vitro pS2 gene expression E-screen	47

Table 1 cont'd: Examples of endocrine disrupting compounds: natural products (phytoestrogens and mycoestrogens) and synthetic chemicals

Compound	Mode of action	Assay	Reference
5.2 Fungicides			
Vinclozolin	antiandrogen	in vitro androgen receptor binding assay, YES	48 19
Dodemorph, Triadimefon, Biphenyl	estrogenic	YES, Vtg – in vitro	43
5.3 Herbicides			
Atrazine	estrogen, antiestrogen	RER (MCF-7-luc), in vivo	23
Simazine	antiestrogen	in vivo	49
Alachlor, Nonachlor	ER agonists	YES, CB-ER, RER (MCF-7-luc)	42
Tributyltins	androgenic	imposex in snails, various in vivo effects in gastropods	25, 50
6. INDUSTRIAL CHEMICALS			
6.1 Phthalates			
Butylbenzylphthalate (BBP)	ER agonist, antiandrogenic activity	in vitro + in vivo, E-screen, YES	25, 39, 51, 19
Dibutylphthalate (DBP)	ER agonist	in vitro + in vivo	25, 51
6.2 Alkylphenols			
Nonylphenol (NP)	ER agonist, estrogenic	RER (MCF-7-luc, ER-CALUX), YES, number of in vitro and in vivo assays, E-screen, Vtg – in vitro	23, 24, 28, 52, 36, 29, 39
Octylphenol (OP)	ER agonist	RER (MCF-7-luc) number of in vitro and in vivo assays	52 39
Butylphenol, Pentylphenol	estrogenic	E-screen	39, 29
Nonylphenol polyethoxylates and polyethoxycarboxylates	ER agonists	number of in vitro and in vivo assays	52
Pentachlorophenol (PCP)	decrease in blood testosterone concentration	in vivo ewes feeding study	53
Bisphenol A (BPA)	ER agonist antiandrogenic activity	RER (MCF-7-luc, ER-CALUX), YES, Vtg-in vitro YES	23, 24 28, 39 19
7. PERSISTENT ORGANIC POLLUTANTS (POPs)			
Polychlorinated dibenzo-p-dioxins (PCDDs)	antiestrogenic – different mechanisms	in vivo + in vitro studies	54
Polychlorinated biphenyls (PCBs)	ER agonists or antagonists or other mechanism – depending on the substitution	RER (transient MCF-7-luc), E-screen, in vivo – vaginal cell cornification in mice	21, 39
Aroclor 121 (PCBs mix), Aroclor 1260	estrogenic, effect on sexual differentiation, gonadal abnormalities	Vtg-in vitro in vivo trout study	39 55
Hydroxy-PCBs	ER agonists or antagonists	RER (MCF-7-luc), E-screen, CB-ER, in vivo – vaginal cell cornification in mice	53, 56, 21, 39
Polycyclic aromatic hydrocarbons (PAHs)	ER agonists – estrogenic, antiestrogenic – different mechanisms	YES, E-screen RER (MCF-7-luc)	57, 58, 59 60
6-Hydroxy – chrysene	antiestrogenic	YES	57
8. HEAVY METALS			
Cations of cadmium, cobalt, copper, mercury, nickel, zinc	depression or increase in testosterone production	in vitro substrate stimulated testosterone production by Leydig cells	61
cadmium	decrease in plasma testosterone and cortisol modification of pituitary hormone secretion	in vivo juvenile rainbow trout exposure in vivo rat feeding exposure	62 63
lead	delayed sexual maturation, suppression of sex steroid biosynthesis	in vivo rat feeding study	64
YES = yeast based recombinant estrogen receptor-reporter assay E-screen = MCF-7 cell proliferation CB-ER = in vitro competitive receptor binding assay RER = in vitro recombinant receptor-reporter cell bioassay VTG-in vitro = in vitro vitellogenin synthesis in cultured male trout hepatocytes			

Table 2: Examples of wild type and recombinant cell lines used for assessment of AhR- and ER-mediated activity

Cell line	Reporter gene	Species of origin	Response measured	Reference
Aryl Hydrocarbon Receptor				
H4IIE		rat	EROD	17, 71
HepG2		human	EROD	82
RLT-W1		trout	EROD	77
PHLC-1		fish	EROD	66, 83
H4IIE = CALUX	luc	rat	luciferase	71, 69, 72
HeLa	luc	human	luciferase	84
HepG2	luc	human	luciferase	69, 79
GPC16	luc	guinea pig	luciferase	69
MLE-BV	luc	mouse		
MCF7, LS180	luc – transient	human		
AHL	luc – transient	hamster		
Hepa1	luc, luc-transient	mouse	luciferase	69, 60, 85
Hepa1	PAP - transient	mouse	PAP	76
HepG2, MCF7		human		
H4IIE		rat		
GPC16		guinea pig		
Hepa1	PAP	mouse	PAP	76
RLT2.0	luc	trout	luciferase	78
Estrogen Receptor				
MCF-7	luc - transient	human	luciferase	60, 9
MCF-7 (MVLN)	luc	human	luciferase	85, 56
T47D (ER-CALUX)	luc	human	luciferase	28

Reporter genes: nothing = wild type cell line, luc = luciferase, PAP = alkaline phosphatase

The character of the dose-response curves for endogenous enzyme activities controlled by the AhR mechanism is biphasic with a decrease in response at greater doses. Some chemicals inducing the cytochrome P4501A1 (CYP1A1) activity can also serve as substrates for this enzyme, so they cause competitive substrate inhibition and reduced activity at greater concentrations [69,70,66]. This problem is avoided in genetically engineered cell lines, where the chemical inducers are not competitive substrates for the transfected reporter enzyme. Genetically engineered cells generally exhibit greater sensitivity, dynamic range and selectivity than their corresponding wild type cells [71,72]. Wild type and recombinant cell lines have been developed mostly for mammal and teleost species. Studies are being conducted to develop cell lines for other species, including amphibians and reptiles. In addition, immortalized (continuous) cell cultures of primary hepatocytes from birds, mammals or fish have been used to measure dioxin-like activity [73,74,75] or xenoestrogenicity [67]. The responsiveness of assays as characterized by maximal fold induction relative to control, sensitivity, detection limit and variability is species- and cell line-specific. Differences among species and tissues in ligand-binding affinity, ligand specificity and physicochemical properties of the receptor have been shown along with significant differences in responsiveness to standard ligands [76]. Observed differences in responsiveness are explained by spe-

cies differences in the level and structure of the receptors and their associated proteins, and/or transacting factors present in each cell line [69,21]. Studies comparing responsiveness among cell lines from different species (mostly mammals and fish) to single compound or mixtures revealed substantial differences between the relative potencies derived from different species [4,77]. Also the time course of response differs among cell lines. Fish cells have been observed to be slower in responsiveness than the mammalian cells [78]. Therefore, the optimum duration of exposure is important to obtain reproducible results and is cell-line specific ranging from 6 hrs to 72 hrs [79].

Both estrogenic and antiestrogenic effects can be assessed with ER-responsive cell lines. Antiestrogenicity can be detected directly by growing cells in medium deficient in 17 β -estradiol (E₂) or by the antagonism of co-administered E₂ [56]. As one of the important mechanisms of antiestrogenicity, modulation of endocrine pathways by AhR agonists has been observed [80]. TCDD and related compounds have been observed to be antiestrogenic in *in vitro* tests but also in some *in vivo* studies [2]. The interactions of the TCDD- and E₂-induced signaling pathways are complex; AhR agonists are antiestrogenic via direct interactions between the nuclear AhR and genomic sequences in flanking regions of E₂-regulated genes [54]. Two way cross-talk be-

tween the intracellular signaling pathways involving AhR agonists and estrogens by mutual inhibition of binding of ER or AhR to DNA has been reported [81].

In extracts containing complex mixtures of compounds, potential cytotoxicity should be evaluated in bioassays with the same cells that were used in receptor mediated effects. This is because the cytotoxic effects could mask potential antiestrogenic or other types of effects. Other mechanisms of antiestrogenicity include:

- 1) competitive binding of the ligand to the ER displacing E_2 ;
- 2) increased E_2 metabolism due to induction of xenobiotic metabolizing enzymes;
- 3) inhibition of E_2 -induced gene expression;
- 4) estrogen receptor down regulation [2].

Anti-/estrogenic potential of some compounds changes depending on the E_2 concentration [56], thus testing only in media without any E_2 does not adequately assess the physiological situation where there is always some E_2 present.

4 Testing of Complex Mixtures with Bioassays

In vitro bioassays have been used to assess TCDD-like and estrogenic activity in a variety of environmental matrices, both abiotic and biotic. Various aquatic samples, such as porewater [72], stream water [83], extracts from waste water treatment plant influent and effluents, sediments [72] or settling particulate matter [87,88] have been analyzed by *in vitro* cell bioassays. Extracts from semi-permeable membrane devices (SPMDs) enabled examination of concentrations of *in situ* bioavailable lipophilic contaminants to which aquatic organisms are exposed [83]. Also sludge [28,86] or atmospheric samples including air particulates [60] and fly ash from incinerators [75] can be assessed after extraction. Paper mill effluent fractions elicited strong TCDD-like and antiestrogenic activity [9], whereas significant estrogenic and TCDD-like activity has been detected in crude extract of inhalable air particulate matter [60] or from diesel exhaust particles [89]. Significant dioxin-like activity has been observed in eggs of birds such as herring gull, cormorant, and great blue heron [65,74] as well as in birds at different stages of development [90]. Among other animals, extracts of fish (white sucker, juvenile whitefish)[17,86] and otter [91] have also been tested. For the biota samples either whole body extracts or more specific tissue extracts, especially livers have been used. An important step is the sample preparation and extraction. Direct water sample or extracts with organic solvents can be used. Solid samples are usually extracted by organic solvents. The solvent of choice needs to be compatible with the cell system, not causing any effect by itself, but enabling distribution of the extracted material to the cells.

Extracts can be cytotoxic, which is caused by some compounds present in complex mixtures. For example, sulfur is a major cytotoxic constituent in sediment extracts, which should be eliminated prior to performing dioxin-like or estrogenic activities. The measurement of cell viability/cytotoxicity is essential in all bioassays dealing with complex mixtures as well as single compounds. Cell bioassays with 96 well plates enable the measurement of several samples at

the same time. In addition, current procedures allow subsequent measurement of viability index, enzyme activity and protein content in the same 96-well plates [15].

5 Estimation of Relative Potencies of Complex Mixtures

The relative potencies of samples are usually calculated as the amount of standard (reference toxicant) giving the same response as the sample, commonly based on the amount of sample needed to produce 50% of the maximal standard response (EC_{50}). The exogenous compound with the greatest known affinity as well as toxicity, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, is used as a standard for AhR-mediated responses. The endogenous substrate 17 β -estradiol (E_2) serves as a standard for ER-mediated activity. Activities of samples are then expressed as bioassay-derived equivalents: dioxin equivalents (TCDD-EQ) or estradiol equivalents (E_2 -EQ) per specified amount of sample. For calculating and comparing the equivalents complete dose-response curves from step-wise diluted extracts and standards should be obtained. This is rather difficult with complex extracts. Common problems encountered when determining the relative potencies of complex mixtures include different efficacy (maximal induced response), non-parallel slopes, cytotoxicity at greater concentrations or insufficient mass of agonists to reach full efficacy or the occurrence of partial agonists that do not attain the maximum possible response. These limitations must be taken into account when calculating the relative potency of the sample. There is always variation in the EC_{50} in replicates measured on different days due to differences in plating density. For some cell lines the normalization for protein content can solve this problem. For endogenous enzyme activities the normalization to protein content is necessary. In some transgenic cell lines the normalization to the amount of protein present has been inadvisable because of increased variability of the normalized results. Protein normalization is not recommended in cell lines used for estrogen-receptor mediated activity, where response induction correlates with estrogen-induced protein synthesis [23].

Some non-active parent compounds can be metabolically activated to potent inducers of receptor-mediated response; alternatively the active compound can be biotransformed to non-active metabolites. For most compounds, the activity of their metabolites is unknown. Some of the cell lines possess metabolic capabilities and upon prolonged duration of exposure they can partly simulate *in vivo* biotransformation of some compounds. This fact can be used analytically by use of selective inhibitors.

6 Mass Balance Calculations

In the mass-balance approach, total activities determined by a bioassay are compared with the sum of the potency of the individual compounds determined by chemical analysis. This strategy has been widely used for dioxin-like compounds [4,5] and recently for estrogenic compounds [92]. Toxic equivalents (TEQs) are calculated by multiplying the relative potency (RP) for the specific assay (if available) or the

international toxic equivalency factor (TEF) by concentration of the specific congener giving total sum toxic equivalents per mass unit. For calculating the TEQs from chemical data effects are assumed to be additive (Equation 1).

$$TEQ = \sum_{i=1}^N CONC. OF COMPOUND_i \times TEF_i \quad (1)$$

TEFs are species-, endpoint- and assay specific determination of potency expressed relative to the standard, they can vary widely depending on the species and endpoint. The relative potencies (RPs) should be used for bioassay-directed mass-balance calculation for complex mixtures, they are specific for studied endpoint and assay [93].

The international TEFs are consensus values, based on many different types of assays [4] including multiple *in vitro* and *in vivo* endpoints for multiple species. TEF values are order-of-magnitude estimates suitable for risk assessment purposes. Because of the differences in RPs among species, specific sets of international TEFs have been established for mammals, fish and birds [4]. Currently TEFs and RPs are available for dioxins, furans, some PCBs and PAHs from a number of assays. There are many compounds with potential AhR-mediated activity for which RPs are unavailable and TEFs have not been established. Therefore those compounds cannot be included in the mass-balance calculations.

Limited data are available for the RPs of estrogenic compounds; RPs have been established only by use of *in vitro* bioassays for a few alkylphenolic compounds and PAHs [23,60]. In this case by calculating the E₂-EQs based on analytical results one can estimate the proportion of the total activity determined by bioassay that is represented by the compounds which have been quantified and have known relative potencies. There are several limitations of calculating TEQs from analytical results:

- 1) RPs or TEFs are available for only limited number of chemicals; for some compounds there are no endpoint-specific nor consensus values for TEFs available;
- 2) the use of TEFs derived for other species, usually from mammals, where the most research has been conducted, to nonmammalian species may not be suitable due to the interspecies differences in sensitivity;
- 3) there may be some compounds not routinely detected whose contribution to the activity would be overlooked;
- 4) application of the additive approach is routinely used in the total activity calculation; potential interactions among compounds in a mixture, such as synergism or antagonism are neglected;
- 5) detailed analysis of trace contaminants require specialized equipment such as HRGC/HRMS (high resolution gas chromatograph/mass spectrophotometer), which is not available in all laboratories and may be prohibitively expensive.

Toxic equivalents estimated based on analytical data are correlated with the bioassay results in some situations, depending on the composition of the complex mixture of compounds in the samples. For biota samples for which we report here, highly significant correlations have been found between bioassay derived EROD activity and instrumentally measured TEQs in extracts of fish or bird samples [65,74,17].

However, toxic activities determined in the bioassays and concentrations of known dioxin-like or xenoestrogenic compounds are sometimes not correlated. For instance, data obtained from bioassays may be an independent parameter that is predictive of ecotoxicological effects. Besides non-additive (synergistic or antagonistic) interactions among individual ligands, differences between TEQs derived in bioassays and those calculated from concentrations of individual compounds may be caused by the following events:

- 1) there are some other active compounds present, which were not identified by the chemical analysis [70];
- 2) non-complete dose responses or cytotoxicity disable accurate estimations of toxic equivalents;
- 3) the RPs or TEFs used may not be appropriate.

7 Fractionation Approach

In vitro bioassays can be used in combination with specific analytical techniques as a bioassay-directed fractionation methodology. This approach provides information needed for monitoring and risk assessment of the compounds with specific modes of action and may lead to identification of novel classes of environmental toxicants [88]. If complex mixtures cause a significant response in a bioassay in order to determine the causes and identify possible sources, the compounds causing the observed response need to be identified. Instrumental analysis could be applied to the entire mixture or sub-fractions. Recommended strategy for toxicants identification and evaluation (TIE) in complex mixtures is shown (\rightarrow Fig. 3). The general steps are:

- 1) Screening of the whole extract – to determine the samples containing significant toxic potencies, which require further chemical analysis. If no significant response is observed, there is no need to conduct expensive, time- and material-consuming chemical analysis. Since the method detection limit is known for the bioassay, an upper limit of concentration of toxic equivalents in the sample can be defined;
- 2) Fractionation of the samples that were active in bioassays and chromatographic analysis can be used to determine the most probable contributors to the total activity;
- 3) Generating the full dose-response relationship of the unfractionated sample or fractions thereof, so that the total activity of the sample can be determined as response equivalents. Calculation of the mass-balance is accomplished by comparing the activity observed in the bioassay with the potential activity based identification and quantification by instrumental analyses. If the values derived from the bioassay and those calculated from the concentrations of compounds and relative potency factors and bioassay results for fractions does not indicate that there were antagonistic effects in the whole extract, it can be concluded that all of the significant contributors to the total complex mixture have been identified. However, if the total activity determined from the bioassay is significantly greater than those predicted from the instrumental analyses, then it can be inferred that there are unidentified compounds or that there is synergism. Again by comparing the activity of the whole extract to

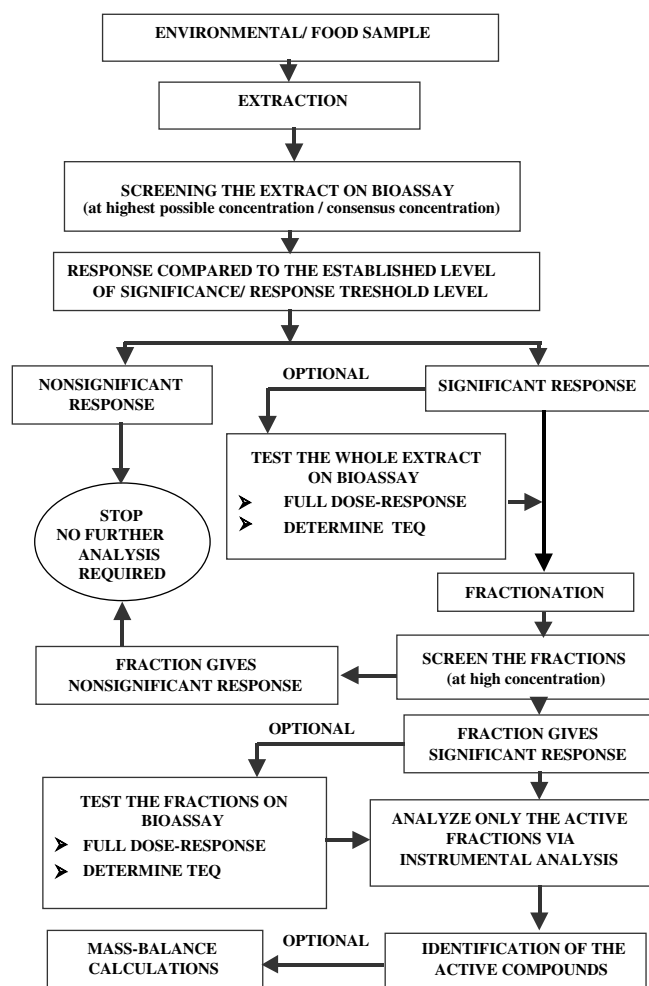


Fig. 3: Screening system: Toxicant identification and evaluation strategy. [For a discussion of different approaches for conducting TIE, see *Exotoxicol. Environ. Safety* **41**, 77-82 (1998)]

that of the various fractions, it is possible to determine if the difference is due to the presence of unidentified compounds or synergism. In our studies, we have found that antagonisms can occur, particularly between non-AhR-active and AhR-active PCB congeners [18].

To apply the mass balance approach with complex mixtures, species- and endpoint-specific RPs/TEFs and especially E_2 -EQs need to be determined. Fractionation of whole extracts into groups of compounds with similar characteristics and subsequent bioassay testing can be useful in determining the most appropriate instrumental analysis that should be applied and can prevent application of non-essential and costly analysis of the fractions with low activity and thus significance [87]. For most compounds, fractionation based on polarity and/or molecular size of the compounds is generally suitable. These characteristics are easily selected for with simple chromatographic techniques. Instrumental analyses can be applied to determine the compounds responsible for the activity observed in each fraction. For instance, if the activity was observed in a more polar fraction normal phase liquid chromatography might be deemed more appropriate than gas chromatography, or derivatization might be deemed appropriate before subsequent analyses.

8 Conclusions

Many studies have demonstrated the utility of bioassays in assessment of receptor-mediated activities of both individual chemicals and complex mixtures. Bioassays can be used for the detection and quantitation of receptor agonists/antagonists in complex mixtures, thus providing a relative measure of bioactive compounds in food, biological, or abiotic samples. Bioassays can also be useful for identification and characterization of novel receptor agonists, for examination of species differences in receptor-mediated responses or effectiveness of remediation procedures designed to decrease specific type of contamination. Bioassays are also useful screening tools for identifying responsible compounds following fractionation of a complex mixture, they enable to prioritize samples which require further investigation. *In vitro* cell bioassays are excellent systems for evaluating the activities of chemicals with specific mode of action. Bioassays, based on *in vitro* responses of cells, including both wild-type or recombinant (genetically modified) cell lines can also be used for assessment of other toxicologically and pharmacologically important chemicals where ligand-dependent induction of gene expression has been demonstrated. Such compounds include xenoandrogens, heavy metals and compounds that can cause induction of peroxisome proliferation.

Generally, bioassay data have great ecotoxicological relevance because they represent an integrated biological response. However, it is necessary to point out disadvantages and limitations of *in vitro* bioassays. Bioassays do not account for the pharmacokinetics, tissue distribution and biotransformation that may occur *in vivo*. If cell lines possess only limited metabolic activities, substances active after bioactivation may not be detected by *in vitro* system [83]. Bioassays do not identify the individual compounds causing the response. Bioassays assess only the activity of compounds that act through a specific receptor-mediated mechanism of action. The non-receptor-mediated responses, such as estrogen-like chemicals acting through different mechanisms, are not taken into consideration.

Acknowledgements

Preparation of the manuscript as well as the research on which it is based was supported by the Czech Ministry of Education (CEZ: J07/98:1410003) and Ministry of Agriculture (MZE-M03-99-01). A Fulbright fellowship to K.Hilscherova is gratefully acknowledged.

References

- [1] GRAY, L.E.; KELCE, W.; WIESE, T.; TYL, R.; GAIDO, K.; COOK, J.; KLINEFELTER, G.; DESAULNIERS, D.; WILSON, E.; ZACHAREWSKI, T.; WALLER, C.; FOSTER, P.; LASKEY, J.; REEL, J.; GIESY, J.; LAWS, S.; MCLACHLAN, J.; BRESLIN, W.; COOPER, R.; DI GIULIO, R.; JOHNSON, R.; PURDY, R.; MIHAICH, E.; SAFE, S.; SONNENSCHN, C.; WELSHONS, W.; MILLER, R.; MCMASTER, S.; COLBORN, T. (1997): Endocrine Screening Methods Workshop Report: Detection of Estrogenic and Androgenic Hormonal and Antihormonal Activity for Chemicals That Act Via Receptor or Steroidogenic Enzyme Mechanisms. *Reproduct. Toxicol.* **11**, 719-750
- [2] GILLESBY, B.E.; ZACHAREWSKI, T.R. (1998): Exoestrogens: Mechanisms of Action and Strategies for Identification and Assessment, *Environ. Toxicol. Chem.* **17**, 3-14

- [3] ANKLEY, G.; MIHAICH, E.; STAHL, R.; TILLITT, D.E.; COLBORN, T.; MCMASTER, S.; MILLER, R.; BANTLE, J.; CAMPBELL, P.; DENSLOW, N.; DICKERSON, R.; FOLMAR, L.; FRY, M.; GIESY, J.; GRAY, L.E.; GUINEY, P.; HUTCHINSON, T.; KENNEDY, S.; KRAMER, V.; LEBLANC, G.; MAYES, M.; NIMROD, A.; PATINO, R.; PETERSON, R.; PURDY, R.; RINGER, R.; THOMAS, P.; TOUART, L.; VAN DER KRAAK, G.; ZACHAREWSKI, T. (1998): Overview of a Workshop on Screening Methods for Detecting Potential (Anti-) Estrogenic/Androgenic Chemicals in Wildlife, *Environ. Toxicol. Chem.* **17**, 68-87
- [4] VAN DEN BERG, M.; BIRNBAUM, L.; BOSVELD, B.T.C.; BRUNSTROM, B.; COOK, P.; FEELEY, M.; GIESY, J.P.; HANBERG, A.; HASEGAWA, R.; KENNEDY, S.; KUBIAK, T.; LARSEN, J.C.; VAN LEEUWEN, F.X.R.; DJIEN LIEM, A.K.; NOLT, C.; PETERSON, R. E.; POELLINGER, L.; SAFE, S.; SCHRENK, D.; TILLITT, D.E.; TYSKLIND, M.; YOUNES, M.; WAERN, F.; ZACHAREWSKI, T. (1998): Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect.* **106**, 775-790
- [5] AHLBORG, U.G.; BROUWER, A.; FINGERHUT, M.A.; JACOBSON, J.L.; JACOBSON, S.W.; KENNEDY, S.W.; KETTRUP, A.A.F.; KOEMAN, J.H.; POIGER, H.; RAPPE, C.; SAFE, S.; SEEGAL, R.F.; TUOMISTO, J.; VAN DEN BERG, M. (1992): Impact of Polychlorinated Dibenzop-dioxins, Dibenzofurans, and Biphenyls on Human and Environmental Health, with Special Emphasis on Application of the Toxic Equivalency Factor Concept. *European J. Pharmacol.* **228**, 179-199
- [6] PETERSON, R.E.; THEOBALD, H.M.; KIMMEL, G.L. (1993): Developmental and Reproductive Toxicity of Dioxins and Related Compounds: Cross-Species Comparisons. *Crit. Rev. Toxicol.* **23**, 283-335
- [7] NEBERT, D.W.; PUGA, A.; VASILIOU, V. (1993): Role of the Ah Receptor and the Dioxin-Inducible [Ah] Gene Battery in Toxicity, Cancer, and Signal Transduction. *Ann. New York Acad. Sci.*; 625-641
- [8] LUCIER, G.W.; PORTIER, CH.J.; GALLO, M.A. (1993): Receptor Mechanism and Dose-Response Models for the Effects of Dioxins. *Environ. Health Perspect.* **1**, 36-44
- [9] ZACHAREWSKI, T.R.; BERHANE, K.; GILLEBY, B.E.; BURNISON, B.K. (1995): Detection of Estrogen- and Dioxin-like Activity in Pulp and Paper Mill Black Liquor and Effluent Using *in vitro* Recombinant Receptor/Reporter Gene Assays. *Environ. Sci. Technol.* **29**, 2140-2146
- [10] SAFE, S.H. (1995): Modulation of Gene Expression and Endocrine Response Pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and Related Compounds. *Pharmac. Ther.* **2**, 247-281.
- [11] DENISON, M.S.; HEATH-PAGLIUSO, S. (1998): The Ah Receptor: A Regulator of the Biochemical and Toxicological Actions of Structurally Diverse Chemicals. *Bull. Environ. Contam. Toxicol.* **61**, 557-568
- [12] HANKINSON, O. (1995): The Aryl Hydrocarbon Receptor Complex. *Ann. Rev. Pharmacol. Toxicol.* **35**, 307-340
- [13] POLAND, A.; KNUTSON, J.C. (1982): 2,3,7,8-tetrachlorodibenzo-p-dioxin and Related Halogenated Aromatic Hydrocarbons: Examination of the Mechanism of Toxicity. *Ann. Rev. Pharmacol. Toxicol.* **22**, 517-554
- [14] LEWIS, D.F.V.; IOANNIDES, C.; PARKE, D.V. (1986): Molecular dimensions of the substrate binding site of cytochrome P-448. *Biochem. Pharmacol.* **35**, 2179-2185
- [15] BLANKENSHIP, A.; KANNAN, K.; VILLALOBOS, S.; VILLENEUVE, D.; FALADYSZ, J.; IMAGAWA, T.; JAKOBSSON, E.; GIESY, J. (2000): Relative Potencies of Halowax Mixtures and Individual Polychlorinated Naphthalenes (PCNs) to induce Ah Receptor Mediated Responses in the Rat Hepatoma H4IIE-luc Cell Bioassay. *Environ. Sci. & Technol.* (In press)
- [16] TILL, M.; BEHNISCH, P.; HAGENMAIER, H.; BOCK, K.W.; SCHRENK, D. (1997): Dioxin-like Components in Incinerator Fly Ash: A Comparison between Chemical Analysis Data and Results from a Cell Culture Bioassay. *Environ. Health Perspect.* **105**, 1326-1332
- [17] VAN DEN HEUVEL, M.R.; MUNKITTRICK, K. R.; VAN DER KRAAK, G.J.; MCMAASTER, M.E.; PORTT, C.B.; SERVOS, M.R.; DIXON, D. J. (1994): Survey of Receiving-Water Environmental Impacts Associated with Discharges from Pulp Mills. 4. Bioassay-derived 2,3,7,8-Tetrachlorodibenzo-p-dioxin Toxic Equivalent Concentration in White Sucker in Relation to Biochemical Indicators of Impact. *Environ. Toxicol. Chem.* **13**, 1117-1126
- [18] SANDERRSON, J.T.; GIESY, J.P. (1998): Wildlife Toxicology, Functional Response Assays. In: Meyers, R.A. (ed.) *Encyclopedia of Environmental Analysis and Remediation*, John Wiley & Sons, Inc.; USA, 5272-5297
- [19] SOHONI, P.; SOTO, J.P. (1998): Several Environmental Oestrogens Are Also Antiandrogens. *J. Endocrinol.* **158**, 327-339
- [20] MACHALA, M.; VONDRACEK, J. (1998): Estrogenic Activity of Xenobiotics. *Vet. Med.-Czech* **10**, 311-317
- [21] Joyeux, A.; Balaguer, P.; Germain, P.; Boussioux, A.M.; Pons, M.; Nicolas, J.C. (1997): Engineered Cell Lines as a Tool for Monitoring Biological Activity of Hormone Analogs. *Anal. Biochem.* **249**, 119-130
- [22] FIELDEN, M.R.; CHEN, I.; CHITTIM, B.; SAFE, S.H.; ZACHAREWSKI, T.R. (1997): Examination of the Estrogenicity of 2,4,6,2',6'-Pentachlorobiphenyl (PCB 104), Its Hydroxylated Metabolite 2,4,6,2',6'-Pentachloro-4-biphenylol (OH-PCB 104), and Further Chlorinated Derivative 2,4,6,2',4',6'-Hexachlorobiphenyl (PCB 155). *Environ. Health Perspect.* **105**, 1238-1248.
- [23] VILLENEUVE, D.; BLANKENSHIP, A.L.; GIESY, J.P. (1998): Interactions Between Environmental Xenobiotics and Estrogen Receptor-Mediated Responses In: Denison, M.S.; Helderich, W.G. (eds.) *Toxicant-receptor interactions*. Taylor and Francis, Philadelphia, PA, USA, 69-99
- [24] GAIDO, K.W.; MCDONNELL, D.P.; KORACH, K.S.; SAFE, S.H. (1997): Estrogenic Activity of Chemical Mixtures: Is there Synergism? CIIT Activities, Chemical Industry Institute of Toxicology **2**, 1-7
- [25] STAHLSCHEMIDT-ALLNER, P.; ALLNER, B.; ROMBKE, J.; KNACKER, T. (1997): Endocrine Disrupters in the Aquatic Environment. *Environ. Sci. Pollut. Res.* **4**, 155-162
- [26] MARKIEWICZ, L.; GAREY, J.; ADLERCREUTZ, H.; GURPIDE, E. (1993): In vitro Bioassays of Non-steroidal Phytoestrogens. *Steroid Biochem. Molec. Biol.* **45**, 399-405
- [27] WANG, C.; MAKELA, T.; HASE, T.; ADLERCREUTZ, H.; KURZER, M.S. (1994): Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. *J. Steroid Biochem. Mol. Biol.* **50**, 205-212
- [28] LEGLER, J.; VAN DEN BRINK, C.; BROWER, A.; VETHAACK, D.; VAN DER SAAG, P.; MURK, T.; VAN DER BURG, B. (1998): Assessment of (Anti)estrogenic compounds using a Stably Transfected Luciferase Reporter Gene Assay in the Human T47-D Breast Cancer Cell Line. *Organohalogen Compounds* **37**, 265-268
- [29] NIMROD, A.C.; BENSON, W.H. (1997): Xenobiotic interaction with and alteration of channel catfish estrogen receptor. *Toxicol. Appl. Pharmacol.* **147**, 381-390
- [30] KUIPER, G.G.; LEMMEN, J.G.; CARLSSON, B.; CORTON, J.C.; SAFE, S.H.; VAN DER SAAG, P.T.; VAN DER BURG, B.; GUSTAFSSON, J.A. (1998): Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinol.* **139**, 10, 4252-4263
- [31] SAFE, S.; GAIDO, K. (1998): Phytoestrogens and Anthropogenic Estrogenic Compounds. *Environ. Toxicol. Chem.* **17**, 119-126
- [32] LE BAIL, J.C.; VARNAT, F.; NICOLAS, J.C.; HABRIUX, G. (1998): Estrogenic and Antiproliferative Activities on MCF-7 Human Breast Cancer Cells by Flavonoids. *Cancer Lett.* **130**, 209-216

- [33] CELIUS, T.; HAUGEN, T.B.; GROTMOL, T. WALTHER, B.T. (1999): A sensitive zongenetic assay for rapid *in vitro* assessment of estrogenic potency of xenobiotics and mycotoxins. *Environ. Health Perspect.* **107**, 63-68
- [34] TAYLOR, C.M.; BLANCHARD, B.; ZAVA, D.T. (1984): Estrogen receptor-mediated and cytotoxic effects of the antiestrogens tamoxifen and 4-hydroxytamoxifen. *Cancer-Res.* **44**, 1409-1414
- [35] RAMKUMART, T.; ADLER, S. (1995): Differential positive and negative transcriptional regulation by tamoxifen. *Endocrinol.* **136**, 536-542
- [36] SHELBY, M.D.; NEWBOLD, R.R.; TULLY, D.B.; CHAE, K.; DAVIS, V.L. (1996): Assessing Environmental Chemicals for Estrogenicity Using a Combination of *In vitro* and *In vivo* Assays. *Environ. Health Perspect.* **104**, 1296-1300
- [37] FAVONI, R.E.; DE CUPIS, A. (1998): Steroidal and Nonsteroidal Oestrogen Antagonists in Breast Cancer: Basic Clin. Appr.; *TiPS* **19**, 406-415
- [38] ROUTLEDGE, E.J.; PARKER, J.; ODUM, J.; ASHBY, J.; SUMPTER, J.P. (1998): Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. *Toxicol. Appl. Pharmacol.* **153**, 12-19
- [39] SOTO, A.M.; SONNENSCHN, C.; CHUNG, K.L.; FERNANDEZ, M.F.; OLEA, N.; SERRANO, F.O. (1995): The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103**, Suppl 7, 113-122
- [40] SUMPTER, J.P.; JOBLING, S. (1995): Vitellogenesis as a Biomarker for Estrogenic Contamination of the Aquatic Environment. *Environ. Health Perspect.* **103**, Suppl.7, 173-178
- [41] KELCE, W.R.; STONE, C.R.; LAWS, S.C.; GRAY, L.E.; KEMPPAINEN, J.A.; WILSON, E.M. (1995): Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. *Nature* **375**, 581-585
- [42] KLOTZ, D.M.; BECKMAN, B.S.; HILL, S.M.; MCLACHLAN, J.A.; WALTERS, M.R.; ARNOLD, S.F. (1996): Identification of environmental chemicals with estrogenic activity using a combination of *in vitro* assays. *Environ. Health Perspect.* **104**, 10, 1084-1089
- [43] SOTO, A.M.; CHUNG, K.L.; SONNENSCHN, C. (1994): The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ. Health Perspect.* **102**, 380-383
- [44] PETIT, F.; LE-GOFF, P.; CRAVEDI, J.P.; VALOTAIRE, Y.; PAKDEL, F. (1997): Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. *J. Mol. Endocrinol.* **19**, 321-335
- [45] ASMATBANU, I.; KALIWAL, B.B. (1997): Temporal effect of methyl parathion on ovarian compensatory hypertrophy, follicular dynamics and estrous cycle in hemicastrated albino rats. *J. Basic Clin. Physiol. Pharmacol.* **8**, 237-254
- [46] CRANMER, J.M.; CRANMER, M.F.; GOAD, P.T. (1984): Prenatal chlordane exposure: effects on plasma corticosterone concentrations over the lifespan of mice. *Environ.Res.* **35**, 204-210
- [47] GO, V.; GAREY, J.; WOLFF, M.S.; POGO, B.G.T. (1999): Estrogenic Potential of Certain Pyrethroid Compounds in the MCF-7 Human Breast Carcinoma Cell Line, *Environ. Health Perspect.* **107**, 173-177
- [48] KELCE, W.R.; MONOSSON, E.; GAMSCSIK, M.P.; LAWS, S.C.; GRAY, L.E. (1994): Environmental Hormone Disruptors: Evidence That Vinclozolin Developmental Toxicity is Mediated by Antiandrogenic Metabolites. *Toxicol. App. Pharmacol.* **126**, 267-285
- [49] TENNANT, M.K.; HILL, D.S.; ELDRIDGE, J.C.; WETZEL, L.T.; BRECKENRIDGE, C.B.; STEVENS, J.T. (1994): Possible antiestrogenic properties of chloro-s-triazines in rat uterus. *J. Toxicol. Environ. Health* **43**, 183-196
- [50] MATTHIESSEN, P.; GIBBS, P.E. (1998): Critical Appraisal of the Evidence for Tributyltin-mediated Endocrine Disruption in Mollusks. *Environ. Toxicol. Chem.* **17**, 37-43
- [51] JOBLING, S.; REYNOLDS, T.; WHITE, R.; PARKER, M.G.; SUMPTER, J.P. (1995): A Variety of Environmental Persistent Chemicals, Including Some Phtalate Plasticizers, Are Weakly Estrogenic. *Environ. Health Perspect.* **103**, 582-587
- [52] SERVOS, M.R. (1999): Review of the Aquatic Toxicity, Estrogenic Responses and Bioaccumulation of Alkylphenols and Alkylphenol Polyethoxylates. *Water Qual.Res.J.Canada* **34**, 123 - 177
- [53] BEARD, A.P.; BARTLEWSKI, P.M.; RAWLINGS, N.C. (1999): Endocrine and reproductive function in ewes exposed to the organochlorine pesticides lindane or pentachlorophenol. *J. Toxicol. Environ. Health.* **56**, 23-46
- [54] SAFE, S.; KRISHNAN, V. (1995): Cellular and Molecular Biology of Aryl Hydrocarbon (Ah) Receptor – Mediated Gene Expression. *Arch. Toxicol.; Suppl.* **17**, 99 -115
- [55] MATTA, M.B.; CAIRNCROSS, C.; KOCAN, R.M. (1998): Possible Effects of Polychlorinated Biphenyls on Sex Determination in Rainbow Trout. *Environ. Toxicol. Chem.* **17**, 26-29
- [56] KRAMER, V.J.; HELFERICH, W.G.; BERGMAN, A.; KLASSON-WEHLER, E.; GIESY, J.P. (1997): Hydroxylated Polychlorinated Biphenyl Metabolites are Anti-estrogenic in a Stably Transfected Human Breast Adenocarcinoma (MCF7) Cell Line. *Toxicol. App. Pharmacol.* **144**, 363-376
- [57] TRAN, D.Q.; IDE, C.F.; MCLACHLAN, J.A.; ARNOLD, S.F. (1996): The Anti-estrogenic Activity of Selected Polynuclear Aromatic hydrocarbons in Yeast Expressing Human Estrogen Receptor. *Biochem.Biophys.Res.Comm.* **229**, 102-108
- [58] CHALOUPEK, K.; KRISHNAN, V.; SAFE, S. (1992): Polynuclear Aromatic Hydrocarbon Carcinogens as Antiestrogens in MCF-7 Human Breast Cancer Cells: Role of the Ah Receptor Carcinogenesis **12**, 2233-2239
- [59] SANTODONATO, J. (1997): Review of the Estrogenic and Antiestrogenic Activity of Polycyclic Aromatic Hydrocarbons: Relationship to Carcinogenicity. *Chemosphere* **34**, 835-848
- [60] CLEMONS, J. H.; ALLAN, L.M.; MARVIN, C.H.; WU, Z.; MCCARRY, B.E.; BRYANT, D.W.; ZACHAREWSKI, T.R. (1998): Evidence of Estrogen- and TCDD-Like Activities in Crude and Fractionated Extracts of PM10 Air Particulate Material Using *In Vitro* Gene Expression Assay. *Environ. Sci. Technol.* **32**, 1853-1860
- [61] LASKEY, J.W.; PHELPS, P.V. (1991): Effect of Cadmium and Other Metal Cations on *in Vitro* Leydig Cell Testosterone Production. *Toxicol. Appl. Pharmacol.* **108**, 296-306
- [62] RICARD, A.C.; DANIEL, C.; ANDERSON, P.; HONTELA, A. (1998): Effects of Subchronic Exposure to Cadmium Chloride on Endocrine and Metabolic Functions in Rainbow Trout *Oncorhynchus mykiss*. *Arch. Environ. Contam. Toxicol.* **34**, 377-381
- [63] LAFUENTE, A.; BLANCO, A.; MARQUEZ, N.; ALVAREZ-DEMANUEL, E.; ESQUIFINO, A.I. (1997): Effects of Acute and Subchronic Cadmium Administration on Pituitary Hormone Secretion in Rat. *Rev. Esp. Fisiol.* **53**, 265-269
- [64] RONIS, M.J.; GANDY, J.; BADGER, T. (1998): Endocrine mechanisms underlying reproductive toxicity in the developing rat chronically exposed to dietary lead. *J. Toxicol. Environ. Health* **54**, 77-99
- [65] TILLITT, D.E.; ANKLEY, G.T.; VERBRUGGE, D.A.; GIESY, J.P.; LUDWIG, J.P.; KUBIAK, T.J. (1991): H4IIE Rat Hepatoma Cell Bioassay-Derived 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Equivalents in Colonial Fish-Eating Waterbird Eggs from the Great Lakes. *Arch. Environ. Contam. Toxicology* **21**, 91-101
- [66] HAHN, M.E.; WOODWARD, B.L.; STEGEMAN, J.J.; KENNEDY, S.W. (1996): Rapid Assessment of Induced Cytochrome P4501A

- Protein and Catalytic Activity in Fish Hepatoma Cells Grown in Multiwell Plates: Response to TCDD, TCDF, and Two Planar PCBs. *Environ. Toxicol. Chem.* **4**, 582-591
- [67] PELISSERO, C.; FLOURIOT, G.; FOUCHER, J.L.; BENNETAU, B.; DUNOGUES, J.; LE GAC, F.; SUMPTER, J.P. (1993): Vitellogenin Synthesis in Cultured Hepatocytes – an *In vitro* Test for the Estrogenic Potency of Chemicals. *J. Steroid Biochem. Molec. Biol.* **44**, 263-272
- [68] ZACHAREWSKI, T.R. (1997): In Vitro Bioassays for Assessing Estrogenic Substances. *Environ. Sci. Technol.* **31**, 613-623
- [69] GARRISON, P.M.; TULLIS, K.; AARTS, J.M.M.J.G.; BROUWER, A.; GIESY, J.P.; DENISON, M.S. (1996): Species-Specific Recombinant Cell Lines as Bioassay Systems for the Detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like Chemicals. *Fund. Appl. Toxicol.* **30**, 194-203
- [70] WILLET, K.L.; GARDINALI, P.R.; SERICANO, J.L.; WADE, T.L.; SAFE, S.H. (1997): Characterization of the H411E Rat Hepatoma Cell Bioassay for Evaluation of Environmental Samples Containing Polynuclear Aromatic Hydrocarbons (PAHs). *Arch. Environ. Contam. Toxicol.* **32**, 442-448
- [71] SANDERSON, J.T.; AARTS, J.M.M.J.G.; BROUWER, A.; FROESE, K.L.; DENISON, M.S.; GIESY, J.P. (1996): Comparison of Ah Receptor-Mediated Luciferase and Ethoxyresorufin-*O*-deethylase Induction in H411E Cells: Implications for Their Use as Bioanalytical Tools for Detection of Polyhalogenated Aromatic Hydrocarbons. *Toxicol. App. Pharmacol.* **137**, 316-325
- [72] MURK, A.J.; LEGLER, J.; DENSON, M.S.; GIESY, J.P.; VAN DE GUCHTE, C.; BROUWER, A. (1996): Chemical-Activated Luciferase Gene Expression (CALUX): A Novel In Vitro Bioassay for Ah Receptor Active Compounds in Sediments and Pore Water. *Fund. Appl. Toxicol.* **33**, 149-60
- [73] KENNEDY, S.W.; LORENZEN, A.; JONES, S.P.; HAHN, M.E.; STEGEMAN, J.J. (1996): Cytochrome P4501A Induction in Avian Hepatocyte Cultures: A Promising Approach for Predicting the Sensitivity of Avian Species to Toxic Effects of Halogenated Aromatic Hydrocarbons. *Toxicol. App. Pharmacol.* **141**, 214-230
- [74] KENNEDY, S.W.; LORENZEN, A.; NORSTROM, R.J. (1996): Chicken Embryo Hepatocyte Bioassay for Measuring Cytochrome P4501A-Based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin Equivalent Concentrations in Environmental Samples. *Environ. Sci. Technol.* **30**, 706-715
- [75] TILL, M.; BEHNISCH, P.; HAGENMAIER, H.; BOCK, K.W.; SCHRENK, D. (1997): Dioxin-like Components in Incinerator Fly Ash: A Comparison between Chemical Analysis Data and Results from a Cell Culture Bioassay. *Environ. Health Perspect.* **105**, 1326-1332
- [76] EL-FOULY, M.H.; RICHTER, C.A.; GIESY, J.P.; DENISON, M.S. (1994): Production of a Novel Recombinant Cell Line for Use as a Bioassay System for Detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like Chemicals. *Environ. Toxicol. Chem.* **10**, 1581-1588
- [77] CLEMONS, J.H.; DIXON, D.J.; BOLS, N.C. (1997): Derivation of 2,3,7,8-TCDD Toxic Equivalent Factors (TEFs) for Selected Dioxins, Furans and PCBs with Rainbow Trout and Rat Liver Cell Lines and the Influence of Exposure Time. *Chemosphere* **34**, 1105-1119
- [78] RICHTER, C.A.; TIEBER, V.L.; DENISON, M.S.; GIESY, J.P. (1997): An In Vitro Rainbow Trout Cell Bioassay for Aryl Hydrocarbon Receptor-Mediated Toxins. *Environ. Toxicol. Chem.* **3**, 543-550
- [79] ANDERSON, J.W.; ZENG, E.Y.; JONES, J.M. (1999): Correlation between Response of Human Cell Line and Distribution of Sediment Polycyclic Aromatic Hydrocarbons and Polychlorinated Biphenyls on Palos Verdes Shelf, California, USA. *Environ. Toxicol. Chem.* **18**, 1506-1510
- [80] NAVAS, J.M.; SEGNER, H. (1998): Antiestrogenic Activity of Anthropogenic and Natural Chemicals. *Environ. Sci. Pollut. Res.* **5**, 75-82
- [81] KHARAT, I.; SAATCIOGLU, F. (1996): Antiestrogenic Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Are Mediated by Direct Transcriptional Interference with the Liganded Estrogen Receptor. *J. Biol. Chem.* **271**, 10533-10537
- [82] NAKAMA, A.; YOSHIKURA, T.; FUKUNAGA, I. (1997): Induction of Cytochrome P450 in HepG2 Cells and Mutagenicity of Extracts of Sediments from a Waste Disposal Site Near Osaka, Japan. *Bull. Environ. Contam. Toxicol.* **59**, 344-351
- [83] VILLENEUVE, D.; CRUNKILTON, R.L.; DEVITA, W.M. (1997): Aryl Hydrocarbon Receptor-Mediated Toxic Potency of Dissolved Lipophilic Organic Contaminants Collected from Lincoln Creek, Milwaukee, Wisconsin, USA, to PLHC-1 (*Poeciliopsis Lucida*) Fish Hepatoma Cells. *Environ. Toxicol. Chem.* **16**, 977-984
- [84] BALAGUER, P.; JOYEUX, A.; DENISON, M.S.; VINCENT, R.; GILLESBY, B.E.; ZACHAREWSKI, T.R. (1996): Assessing the Estrogenic and Dioxin-like Activities of Chemicals and Complex Mixtures Using *In Vitro* Recombinant Receptor-Reporter Gene Assay. *Can. J. Physiol. Pharmacol.* **74**, 216-222
- [85] PONS, M.; GAGNE, D.; NICOLAS, J.C.; MEHTALI, M. (1990): A new cellular model of response to estrogens: a bioluminescent test to characterize (anti) estrogen molecules. *Biotechniques* **9**, 450-459
- [86] KOISTINEN, J.; SOIMASUO, M.; TUKIA, K.; OIKARI, A.; BLANKENSHIP, A.; GIESY, J.P. (1998): Induction of EROD Activity in Hepa-1 Mouse Hepatoma Cells and Estrogenicity in MCF-7 Human Breast Cancer Cells by Extracts of Pulp Mill Effluents, Sludge, and Sediments Exposed to Effluents. *Environ. Toxicol. Chem.* **17**, 1499-1507
- [87] ENGWALL, M.; BROMAN, D.; DENCKER, L.; NAF, C.; ZEBUHR, Y.; BRUNSTROM, B. (1997): Toxic Potencies of Extracts from Sediments and Settling Particulate Matter Collected in the Receptant of a Bleached Pulp Mill Effluent before and after Abandoning Chlorine Bleaching. *Environ. Toxicol. Chem.* **16**, 1187-1194
- [88] BRUNSTROM, B.; BROMAN, D.; DENCKER, L.; NAF, C.; VEJLENS, E.; ZEBUHR, Y. (1992): Extracts from Settling Particulate Matter Collected in the Stockholm Archipelago Waters: Embryo lethality, Immunotoxicity and EROD, inducing Potency of Fractions Containing Aliphatics/Monoaromatics, Diaromatics or Polyaromatics. *Environ. Toxicol. Chem.* **11**, 1441-1449
- [89] MEEK, M.D. (1998): Ah Receptor and Estrogen Receptor-Dependent Modulation of Gene Expression by Extracts of Diesel Exhaust Particles. *Environ. Res.* **79**, 114-121
- [90] JONES, P.D.; GIESY, J.P.; NEWSTED, J.L.; VERBRUGGE, D.A.; LUDWIG, J.P.; LUDWIG, M.E.; AUMAN, H.J.; CRAWFORD, R.; TILLITT, D.E.; KUBIAK, T.J.; BEST, D.A. (1994): Accumulation of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Equivalents by Double-Crested Cormorant (*Phalacrocorax auritus*, Pelicaniformes) Chicks in the North American Great Lakes. *Ecotoxicol. Environ. Safety* **27**, 192-209
- [91] MURK, A.J.; LEONARD, B.E.G.; VAN HATTUM, B.; LUIT, R.; VAN DER WEIDEN, M.E.J.; SMIT, M. (1998): Application of Biomarkers for Exposure and Effect of Polyhalogenated Aromatic Hydrocarbons in Naturally Exposed European Otters (*Lutra lutra*). *Environ. Toxicol. Pharmacol.* **6**, 91-102
- [92] SAFE, S. (1995): Environmental and Dietary Estrogens and Human Health: Is There a Problem? *Environ. Health Perspect.* **103**, 346-351
- [93] VILLENEUVE, D.; RICHTER, C.A.; GIESY, J.P. (1999): Rainbow Trout Cell Bioassay Derived TEFs for Halogenated Aromatic Hydrocarbons: A Comparison and Sensitivity Analysis. *Environ. Toxicol. Chem.* **18**, 879-888

Additional Literature

- HOFMAIER, A.; MARKMANN, A.; LEHNARDT, R.; SCHRAMM, K.-W.; KAUNE, A.; KETTRUP, A. (1998): Measuring TCDD Equivalents (TEQ) in Emission Samples from a Plant, Utilising Secondary Aluminium and Environmental Samples with a Bioassay. In: *Dioxin '98 - 18th Symposium on Halogenated Environmental Organic Pollutants/Formation and Sources, Transport and Fate* (Eds.: N. Johansson et al.). Stockholm: Swedish Environmental Protection Agency, Organohalogen Compounds 36, 187-191
- HOFMAIER, A.M.; SCHWIRZER, S.M.G.; WIEBEL, F.J.; SCHRAMM, K.-W.; WEGENKE, M.; KETTRUP, A. (1998): Bioassay zur Bestimmung von TCDD-Toxizitätsäquivalenten (TEQ) von Umweltproben und Reststoffen. *UWSF - Z. Umweltchem. Ökotox.* 11, 2-8 (1998)
- KLIMM, C.; HOFMAIER, A.M.; SCHRAMM, K.-W.; KETTRUP, A. (1999): Using TEF Concept for Assessing Toxic Potency of Polycyclic Aromatic Hydrocarbons in Industrial Samples. *Organohalogen Compounds* 40, 39-42
- LI, W.; WU, W.Z.; BECK, B.; SCHRAMM, K.-W.; KETTRUP, A. (1999): A New Enzyme Immunoassay for PCDD/F TEQ Screening In Environmental Samples: Comparison to Micro-Erod Assay and to Chemical Analysis. *Chemosphere* 38, 3313-3318
- LI, W.; WU, W.Z.; SCHRAMM, K.-W.; KETTRUP, A. (1999): Toxicity of Mixtures of Polychlorinated Dibenzo-*p*-dioxins, Dibenzofurans, and Biphenyls Determined by Dose-Response Curve Analysis. *Bull. Environ. Toxicol.* 62, 539-546
- REHMANN, K.; RUDZKI, M.; SCHRAMM, K.-W.; KETTRUP, A. (1999): Erfahrungen mit einem Hefe-Zest zum Nachweis von Östrogenrezeptor-aktivierenden Substanzen in Umweltproben. *Ökotoxikologie* (Ed. Oehlmann, Markert), ISBN 3-609-68370-8, ecomed, 538-545
- REHMANN, K.; SCHRAMM, K.-W.; KETTRUP, A. (1999): Applicability of a Yeast Oestrogen Screen for the Detection of Oestrogen-Like Activities in Environmental Samples. *Chemosphere* 38, 3303-3312
- REHMANN, K.; SCHRAMM, K.-W.; KETTRUP, A. (1999): Soot as a Source of Estrogen- and Androgen Receptor Activating Compounds. *Organohalogen Compounds* 42, 105-108
- REHMANN, K.; WU, W.Z.; SCHRAMM, K.-W.; KETTRUP, A. (1999): Xenoestrogens in Unexpected Places - First Results from the Application of a Yeast Oestrogen Assay on Non Aqueous Environmental Samples. *Proceedings Industrial Toxicology, ISSN 1335-3160*, 129-131
- SCHRAMM, K.-W.; HOFMAIER, A.; KLOBASA, O.; KAUNE, A.; KETTRUP, A. (1999): Biological In Vitro Emission Control. *J. of Analytical and Applied Pyrolysis* 49, 199-210
- SCHRAMM, K.-W.; HOFMAIER, A.; KLOBASA, O.; REHMANN, K.; KAUNE, A.; WU, W.Z.; KETTRUP, A. (1999): Biological In Vitro Investigation of Industrial (and Environmental) Samples. *Proceedings Industrial Toxicology, ISSN 1335-3160*, 22-30
- SCHWIRZER, S.M.G.; HOFMAIER, A.M.; KETTRUP, A.; NERDINGER, P.E.; SCHRAMM, K.-W.; THOMA, H.; WEGENKE, M.; WIEBEL, F.J. (1998): Establishment of a Simple Cleanup Procedure and Bioassay for Determining 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Toxicity Equivalents of Environmental Samples. *Ecotoxicology and Environmental Safety* 41, 77-82
- WU, W.Z.; SCHWIRZER, S.M.G.; SCHRAMM, K.-W.; WIEBEL, F.J.; XU, Y.; ZHANG, Y.Y.; YEDILER, A.; KETTRUP, A. (1996): Rapid Bioassay as Indicator of Potentially Harmful Effects for Dioxin-like Compounds in Sample of Ya-Er Lake, China: Requirements for Clean-up and Comparison to Chemical Analysis. *Fresenius Envir. Bull.* 5, 374-379

Received: September 14th, 1999

Accepted: February 9th, 2000

Online-First: February 28th, 2000

News & Views

EPA – Draft Dioxin Reassessment

<http://www.epa.gov/ncea/pdfs/dioxin/dioxreass.htm>

The U.S. Environmental Protection Agency's (EPA) is progressing toward completion of its comprehensive reassessment of dioxin science entitled, "*Exposure and Human Health Reassessment of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) and Related Compounds.*" The next major step in the reassessment process is submitting 2 key chapters to independent peer review, specifically a new chapter entitled *Toxicity Equivalence Factors (TEF) for Dioxin and Related Compounds* and the revision of the *Integrated Summary and Risk Characterization for 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) and Related Compounds*. It is important to note that EPA will not use the conclusions of the draft dioxin reassessment for regulatory purposes until the science peer reviews are completed.

EPA has prepared five information sheets to provide background information on the reassessment, the development of dioxin strategy, and EPA's ongoing dioxin regulatory programs. The information sheets are listed below with a short description of their purpose and contents. All of these information sheets, as well as the draft reassessment documents, are available on the Internet at <http://www.epa.gov/ncea/dioxin.htm>

1. Dioxin: Summary of the Dioxin Reassessment Science
This information sheet provides an abbreviated summary of the science in the draft dioxin reassessment. A more in-depth discussion can be found in the companion piece entitled *Dioxin: Scientific Highlights from Draft Reassessment (2000)* (Information Sheet 2).
2. Dioxin: Scientific Highlights from Draft Reassessment (2000).
This information sheet provides a more in depth discussion of the reassessment science in the draft documents.

3. Dioxin: Reassessment Process - EPA is Moving Toward Completion of the Dioxin Reassessment.

This information sheet briefly describes the process that EPA has used in developing the final reassessment and includes a discussion of the remaining steps of peer review and public comment that are being taken to insure the quality of the final product.

4. Dioxin: Summary of Major EPA Control Efforts.

This information sheet provides background information on the broad set of dioxin regulatory and risk management activities that EPA is pursuing through its major programs areas. Collectively, these actions address all of the major industrial dioxin sources so far identified in the reassessment. Many of these EPA dioxin control efforts were initiated while the dioxin reassessment was being developed.

5. Dioxin: Development of EPA Cross Media Dioxin Strategy.

This information sheet describes a parallel activity to the reassessment; the development of an agency-wide dioxin strategy. This strategy will integrate EPA's diverse set of dioxin activities into a comprehensive program that is consistent with and responsive to the findings of the dioxin reassessment. It is important to note that EPA will not use the conclusions of the draft dioxin reassessment for regulatory purposes until the science peer reviews are completed.

Federal Register Notice

This June 12 FR notice has given public notice of the upcoming external peer review meeting and announced the availability of the draft documents and the beginning of the public comment period.