Development and application of *in vitro* and *in vivo* reporter gene assays for the assessment of (xeno-)estrogenic compounds in the aquatic environment

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Development and application of *in vitro* and *in vivo* reporter gene assays for the assessment of (xeno-)estrogenic compounds in the aquatic environment

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Proefschrift

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for my parents

Stellingen

1. Met behulp van de nieuwe *in vitro* en *in vivo* reporter gen assays, die kunnen worden ingezet voor het snel determineren van de aanwezigheid van biologisch-actieve oestrogene stoffen, is aangetoond dat op enkele plaatsen in het Nederlandse aquatische milieu al niveaus van (xeno-) oestrogenen voorkomen, die zeer waarschijnlijk ongewenste effecten op de voortplanting van vissen zouden kunnen veroorzaken. *(dit proefschrift)*

2. Terwijl persistente stoffen *in vivo* hogere oestrogene activiteit vertonen dan voorspeld wordt met *in vitro* bioassays, kan de oestrogene potentie van snel metaboliseerbare stoffen mogelijk overschat worden door *in vitro* bioassays. *(dit proefschrift)*

3. De synergistische effecten van gecombineerde blootstelling aan de pesticiden paraquat en maneb op het dopamine systeem in de hersenen van de rat kan betekenen, dat blootstelling aan mengsels van pesticiden een bijdrage zou kunnen leveren aan het ontstaan van de ziekte van Parkinson. *(Thiruchelvam, M. et al. (2000) J. Neuroscience, 20, 9207-9214)*

4. Subtiele ecologische-relevante effecten, zoals verlengde metamorfose bij kikkers door PCB-blootstelling, worden niet aangetoond met klassieke toxicologische toetsten. *(naar: Gutleb, A.C. et al. (1999) Environ. Toxicol. Pharmacol. 8, 1-14)*

5. De aanwezigheid van lage niveaus van farmaceutica in het milieu is zorgwekkend, omdat deze stoffen continu terechtkomen in oppervlaktewater via rioolwaterzuivering effluënten en bovendien zeer weinig bekend is over de aquatische toxiciteit van deze stoffen. *(Daughton, C.G. and Ternes, T.A. (1999) Environ. Health Perspect., 107, 907-938)*

6. Met haar plannen om de fok van zogenaamde "agressieve" hondensoorten te verbieden legt de Nederlandse overheid de verantwoordelijkheid voor deze problematiek bij de hond in plaats van bij de eigenaar.

7. De werkelijke morele beproeving van de mens, de meest essentiële (zo diep opgeborgen dat die zich aan onze blik onttrekt), berust op zijn verhouding tot wie aan hem zijn overgeleverd: de dieren. *(Kundera, M. (1983) "De ondraaglijke lichtheid van het bestaan")*

8. Buitenlanders zouden het beste in Nederland kunnen integreren door drie dingen te leren: de Nederlandse taal, fietsen en vergaderen. *(naar: Van Vree, W. (1994) "Nederland als vergaderland")*

Stellingen behorend bij het proefschrift *"Development and application of in vitro and in vivo reporter gene assays for the assessment of (xeno-)estrogenic compounds in the aquatic environment"* door Juliette Legler, Wageningen, 27 februari 2001.

Propositions

1. Application of the newly developed *in vitro* and *in vivo* reporter gene assays, which can be used to rapidly determine the presence of biologically-active estrogenic compounds, has shown that in some localised areas in the Dutch aquatic environment, fish may be exposed to (xeno-)estrogens at levels that may impact their reproductive success. *(this dissertation)*

2. While persistent compounds may be more estrogenic *in vivo* than predicted by *in vitro* assays, the estrogenic potency of rapidly metabolised compounds may be overestimated by *in vitro* assays. *(this dissertation)*

3. The synergistic effects of combined exposure to the pesticides paraquat and maneb on the brain dopaminergic system in the rat suggests that exposure to mixtures of pesticides may play a role in the development of Parkinson's disease. *(Thiruchelvam, M. et al. (2000) J. Neuroscience, 20, 9207-9214)*

4. Subtle, ecologically relevant effects, such as prolonged metamorphosis in PCB-exposed frogs, are not detected with classical toxicological tests. *(based on: Gutleb, A.C. et al. (1999) Environ. Toxicol. Pharmacol. 8, 1-14)*

5. The presence of low levels of pharmaceuticals in the environment is alarming, as these compounds are continually introduced to surface waters via sewage treatment effluent and very little is known of their aquatic toxicity. *(Daughton, C.G. and Ternes, T.A. (1999) Environ. Health Perspect., 107, 907-938)*

6. By outlawing the breeding of so-called "aggressive" dog-breeds, the Dutch government is placing the burden of responsibility for this issue upon the dog instead of the owner.

7. Mankind's true moral test, its fundamental test (which lies deeply buried from view), consists of its attitude towards those who are at its mercy: animals. *(Kundera, M. (1983) "The unbearable lightness of being")*

8. Foreigners would integrate best in The Netherlands by learning three things: the Dutch language, bicycling, and "meeting" skills. *(based on: Van Vree, W. (1994) "The Netherlands as a meeting-place")*

Propositions belonging to the PhD dissertation "*Development and application of in vitro and in vivo reporter gene assays for the assessment of (xeno-)estrogenic compounds in the aquatic environment*" by Juliette Legler, Wageningen, February 27, 2001.

Contents

Chapter 1

General Introduction

The research presented in this thesis describes the development of new bioassays to determine the exposure to and early effects of substances with an estrogenic mode of action. An *in vitro* reporter gene assay using a human breast cancer cell line, and an *in vivo* reporter gene assay using stably transformed (transgenic) zebrafish, were developed. Special emphasis was placed on application of the assays to environmental mixtures to monitor levels of biologically active estrogenic substances in the aquatic ecosystem.

Endocrine disruption: a "stolen future" for humans and wildlife?

In 1992, Carlsen, Skakkebaek and colleagues published a controversial study reporting that a significant, worldwide decrease in sperm quality has occurred over the past 50 years. In addition to decreased sperm counts, increased incidence of testicular cancer and cryptorchidism were reported (Giwercman *et al.*, 1993). Evidence in wildlife species that deleterious effects on male reproductive health were occurring also mounted. In Florida panthers, males were found with low sperm counts and cryptorchidism (Facemire *et al.*, 1995). Male alligators in Lake Apopka in Florida demonstrated an array of reproductive abnormalities, including reduced penis size, abnormal testis morphology and abnormal testicular steroidogenesis (Guillette *et al.*, 1994). In seagulls, altered sex ratio and feminization of sexual behaviour in males was observed (Fry and Toone, 1981). Wild male fish captured in waters receiving sewage effluents showed feminized gonads and production of a yolk protein, which normally is produced in females (see below). These alarming reports lead to the hypothesis that declines in sperm counts and related disorders of the male reproductive system in both humans and wildlife could have arisen because of exposure of the developing fetus or during neonatal life to estrogens (Sharpe and Skakkebebaek, 1993; Colborn *et al.*, 1993). One potential source of increased estrogen exposure was via chemicals in the environment that can mimic natural estrogens, the so-called xeno-estrogens. The term "endocrine disruption" was born. Endocrine disruption has been officially defined as process by which an exogenous substance "causes adverse health effects in an intact organism or its progeny consequent to changes in endocrine function" (European Commission, 1996). This wide definition includes not only the estrogenic substances, but also all substances that can effect endocrine function via interference with hormone (e.g. androgen, thyroid, retinoid or progesterone) pathways.

The reports of endocrine disruption in humans and wildlife (recently reviewed by Golden *et al.*, 1998; Tyler *et al.*, 1998; Vos *et al.*, 1999) did not go unnoticed by the media and resulted in a shock-wave of concern in the general public. Numerous newspaper articles as well as television reports (e.g. the BBC documentary "Assault on the Male" in 1993) raised public awareness, culminating in 1996 with the publication of the book "Our Stolen Future" by Colborn and colleagues. In response to the heightened public concern, research efforts were launched internationally to test the hypothesis that environmental chemicals can disrupt male reproductive function. In fact, very little evidence actually existed to unequivocally demonstrate that environmental substances can interfere with endocrine processes, with the exception of the effects of DES on male offspring (reviewed by Cheek and Maclachan, 1998). Regulatory agencies worldwide, including the European Chemical Industry Council (CEFIC), the Organization of Economic Cooperation and Development (OECD) and the U.S. Environmental Protection Agency, launched ambitious programs to test existing and new chemicals for their potential to disrupt endocrine systems. In The Netherlands, The Health Council published advisory reports on the effects of endocrine-disrupting chemicals in humans (Health Council, 1997) and ecosystems (Health Council, 1999), and a Netherlands Research Platform on Endocrine-Disrupting Compounds (Vethaak *et al.*, 2000a) was initiated. One general theme has arisen from these regulatory initiatives; there is a definite need for the

development of rapid new biologically relevant methods to test chemicals for endocrine-disrupting potential (see below).

Evidence of estrogenic substances in the aquatic environment

One of the most convincing lines of evidence for endocrine disruption stems from effects observed in the aquatic environment. In wild fish populations, intersex (defined as the simultaneous presence of both male and female gonadal characteristics) and testis abnormalities have been found in a high proportion of male fish sampled in rivers, estuaries and coastal waters in the U.K. (Jobling *et al.*, 1998; Lye *et al.*, 1997; Allen *et al.*, 1999) as well as in localized freshwater sites in The Netherlands (Vethaak, 2000). The observed effects on male fish gonads have been associated with exposure to environmental levels of natural, synthetic and xenobiotic chemicals (collectively, "(xeno-)estrogens") in the aquatic environment that can mimic the action of the female hormone estrogen, thereby disturbing endocrine balance and causing feminising effects in the developing male gonads. Accordingly, male rainbow trout maintained in waters receiving wastewater treatment plant (WTP) effluents synthesized high levels of the estrogen-inducible yolk precursor protein vitellogenin (VTG) (Purdom *et al.*, 1994; Harries *et al.*, 1996) and had smaller testis than those of fish collected from reference sites (Harries *et al.*, 1997). Laboratory exposure to (xeno-)estrogenic compounds has resulted in induced VTG (Jobling *et al.*, 1996; Routledge *et al.*, 1998), inhibited testicular growth (Jobling *et al.*, 1996; Panter *et al.*, 1998), testis abnormalities (Miles-Richardson *et al.*, 1999) and formation of intersex gonads (Gimeno *et al.*, 1996; Gray and Metcalfe 1997), suggesting that indeed feminization of fish in the wild may be caused by (xeno-)estrogens.

Role of estrogens in mammalian and fish physiology

Estrogens are steroid hormones made primarily in the female ovaries. Estrogens are found in greater amounts in females than males and are thus referred to as the female hormones. In all vertebrates, estrogens play an important role in many reproductive and developmental processes as they influence growth, development and behaviour (puberty), regulate reproductive cycles (menstruation, pregnancy) and affect many other body parts (bones, skin, arteries, the brain). Recently, estrogens have been shown to be essential for male fertility as well (Hess *et al.*, 1997). 17β-estradiol (E2; structure pictured in Figure 1) is the major endogenous estrogen.

In fish, steroid hormones are important for the sexual differentiation of the gonads, though the mechanisms by which sexual development is controlled have not been clearly elucidated. The critical role of steroids is suggested by the observation that the expected sexual phenotype (based on genotype) can be reversed by the administration of exogenous androgens or estrogens during the labile period of sexual development. Indeed, the administration of estrogens during the sexual differentiation of male fish results in varying degrees of intersex in a variety of fish species (Yamamoto, 1969; Hunter and Donaldson, 1983). In addition to their role in influencing sexual differentiation, estrogens play a crucial

role in the synthesis of the yolk-precursor protein vitellogenin in fish. Increasing E2 concentrations during sexual maturation of female fish stimulates the synthesis of vitellogenin by the liver, which in turn is take up growing ooctyes and stored as yolk (Sumpter and Jobling, 1995).

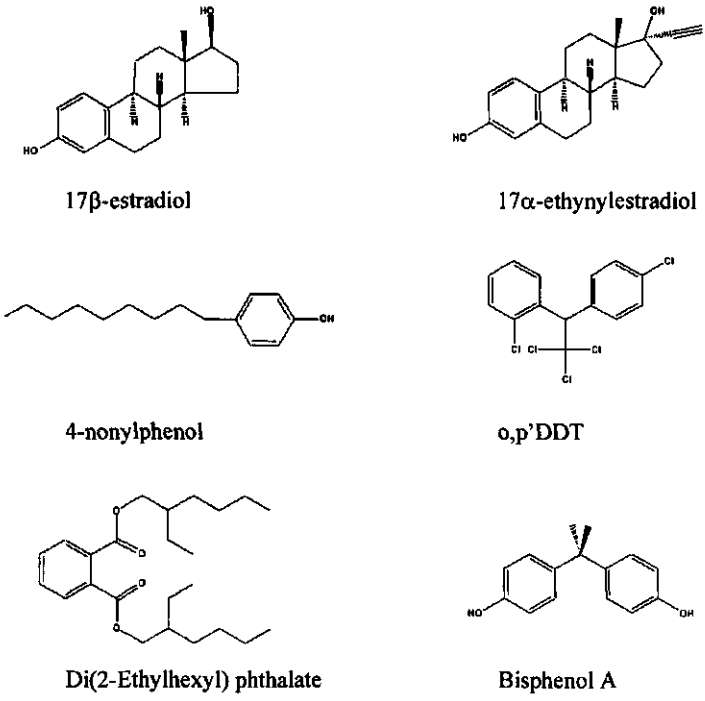

Figure 1: Structures of natural, synthetic and xenobiotic estrogenic chemicals.

Cellular mechanism of action of estrogens

Estrogens are transported through the blood mainly bound to sex hormone binding globulins. Free, non-bound estrogens can exert their action through diffusing through cell membranes and binding estrogen receptors (ER). In addition to the two known ER subtypes (ERα and ERβ), recently a third form was identified in the marine fish Atlantic croaker (ERγ; Hawkins *et al.*, 2000). These three ER subtypes differ in amino acid sequence, as well as tissue distribution, ligand binding affinities and transactivation properties. ERs are found in many tissues, including reproductive organs and accessory sex organs, brain, bone and liver. Inactive ERs exist in large complexes associated with heat shock proteins (Figure 2). Upon

binding of estrogens to the ER, the heat shock proteins disassociate, inducing a conformational change that activates the receptor. Following dimerization of two ER-ligand complexes, binding to estrogen response elements (ERE) of genes in the nucleus takes place. This binding stabilises the binding of transcription factors involved in gene activation and transcription (Figure 2). Gene transcription can be modified by cellular coactivators, repressors and modulators (Stoney, 1996). Following transcription, mRNA is translated into protein by ribosomes. By inducing the synthesis of new proteins that alter cellular functions, estrogens can have profound effects on cell function and physiology (Ing and O'Malley, 1995).

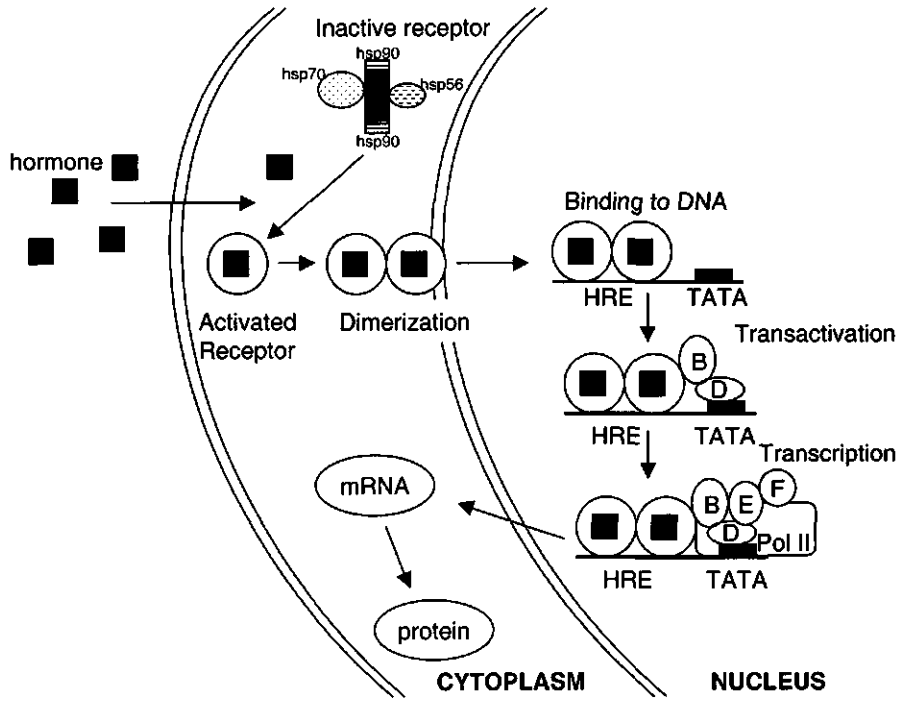

Figure 2: The mechanism of steroid hormone receptor action in the cell. Inactive receptors are associated with heat shock proteins (hsp). Hormones freely enter the cell and bind to inactive receptor, inducing hsp dissociation and conformational change. Activated dimers bind to hormone response elements (HRE) of genes in the nucleus. Transcriptional factors (B,D,E,F) and RNA polymerase II (Pol II) are recruited for transcription. Messenger RNA is subsequently translated into proteins. These new proteins may alter cell function and physiology (adapted from Ing and O'Malley, 1995).

Natural, synthetic and xeno-estrogens

In addition to the endogenous estrogens such as estradiol, estrone and estriol, other compounds found in the environment can mimic estrogens by binding to estrogen receptors and influence estrogen signalling pathways. These compounds include natural chemicals such as the mycoestrogens and phytoestrogens, as well as synthetic estrogens such as the anti-conception pill component ethynylestradiol (pictured in Figure 1) and a number of xenobiotic chemicals (xeno-estrogens). Some examples of xeno-estrogenic compounds are given in Figure 1, and include the organochlorine pesticide o,p'DDT, the industrial surfactant 4-nonylphenol, plasticizers such as di-(2-ethylhexyl)phthalate (DEHP) and Bisphenol A, a compound used in the manufacture of polycarbonate. Some compounds have also been identified with an anti-estrogenic mode of action; that is, they can block, prevent and/or alter binding to estrogen receptors. More detailed lists of phyto- and mycoestrogens, as well as suspected xeno-estrogenic chemicals are provided in Colborn *et al.*, 1993 and Tyler *et al.*, 1998. Recently, the Health Council of The Netherlands reported 34 groups of (xeno-) estrogenic chemicals as potential hormone disruptors which may form a risk for ecosystem health in the Netherlands (Health Council, 1999).

Bioassays for the detection of biologically active (xeno-)estrogens

The large difference in chemical structures of xeno-estrogenic compounds makes prediction of estrogenic activity based on structure difficult. However, xeno-estrogens share a common mechanism of action, namely binding to the estrogen receptor and induction of the ER signal transduction pathway. They also share a common estrogen-mediated biological effect in an intact organism, such as feminization of male gonads or effects on reproduction. Biological test systems or "bioassays" which make use of this common mechanism of action can provide a very useful means of measuring the biological potency of a suspected (xeno-) estrogen or mixture of xeno-estrogens.

A number of *in vitro* bioassays have been developed to screen substances for (anti-) estrogenicity, including competitive ligand binding assays, cell proliferation assays, recombinant receptor/reporter gene assays (see review by Zacharewski, 1997). However, most of these assays suffer from a number of drawbacks that limit their usefulness as a screen for (anti-)estrogenic activity (Zacharewski, 1997). However, recombinant reporter gene assays based on stably transfected cell lines can provide one of the most specific, responsive and biologically relevant means to screen substances for potential anti-estrogenic as well as estrogenic effects with a high throughput potential. This type of assay is based on the receptor-mediated mechanism of action of estrogens and reporter gene expression is a culmination of the molecular cascade of events involved in receptor transactivation. The stably transfected Ah receptor-mediated CALUX (chemical activated luciferase gene expression) assay is an example of an *in vitro* reporter gene assay that has been developed as a sensitive and rapid method to determine the potency of individual congeners and mixtures of dioxin-like compounds in environmental matrices (Murk *et al.*, 1996).

Despite the usefulness of *in vitro* reporter gene assays as tools to rapidly identify (mixtures of) substances with estrogenic potency, prediction of effects of a suspected estrogen in an intact organism is difficult. This is a consequence of the lack of toxicokinetics in the *in vitro* system. For this reason, the use of *in vivo* bioassays has been widely recommended to determine the biological effects of (xeno-)estrogenic compounds (Kavlock *et al.*, 1996; Ankley *et al*, 1998). To assess effects of (xeno-)estrogenic compounds in the aquatic ecosystems, the use of bioassays with fish are warranted. *In vivo* bioassays with teleost fish, including short-term lethality, early life-stage and partial and full life-cycle studies, are already required for regulation purposes by various national and international regulatory agencies, such as the Organization of Economic Cooperation and Development. Because these bioassays can be relatively non-specific in terms of an estrogenic mechanism of action, a number of test systems have been developed to more specifically detect estrogen disrupting chemicals. These included vitellogenin induction, binding of chemicals to fish receptors, or alterations in sexual differentiation (reviewed by Ankley *et al.*, 1998). Some of the disadvantages of these estrogen-specific assays is that they require species-specific antibody development (in the case of vitellogenin), long term exposure regimes, or histopathological expertise. No *in vivo* bioassays exist to rapidly determine the exposure and effects of (xeno-) estrogens in sensitive target tissues and critical life stages of fish.

Outline of thesis

The aim of the research described in this thesis is to develop and apply novel bioassays to rapidly detect chemicals with an estrogenic mode of action. These bioassays were developed to determine the presence of biologically active (xeno-)estrogens in the environment and the estrogenic effects of such exposure on aquatic organisms. Two new bioassays were developed: an *in vitro* estrogen receptor-mediated chemical activated luciferase gene expression (ER-CALUX) assay; and an *in vivo* reporter gene assay using transgenic zebrafish. In both assays, an estrogen-responsive luciferase reporter gene has been stably introduced. Exposure of cells or transgenic zebrafish to (xeno-)estrogens which have an estrogenic mode of action results in reporter gene induction which can be easily measured in cell/tissue lysates.

The development of the ER-CALUX assay is described in Chapter 2. The characterization of the ER-CALUX assay using a range of natural, synthetic and xeno-estrogens is shown in Chapter 2-4. Complex mixtures of (xeno-)estrogens as present in sediments (Chapter 3 and 4), water and biota (Chapter 4) and WTP effluent (Chapter 6) from various locations in The Netherlands were tested for total biologically active estrogenic potency. In addition, the ER-CALUX assay was applied to determine estrogenic potency of (metabolites of) human urine and male fish bile as a possible biomarker for internal dose of (xeno-)estrogens (Chapter 4).

The development of the transgenic zebrafish assay as a rapid *in vivo* test system for (xeno-)estrogens is described in Chapter 5. The zebrafish, *Danio rerio*, was chosen as a model fish species because it has been widely used for toxicological research for decades (reviewed in Laane *et al.*, 1977; Lele and Krone, 1996). This small tropical cyprinid was ideal for this

study because it has a short life cycle and provides large number of eggs. In addition, the genetics underlying zebrafish development and reproduction have been well documented (reviewed by Haffter and Nusslein-Volhard, 1996). Transgenic zebrafish had been already produced at the moment of start of this project (Stuart *et al.*, 1988, reviewed in Lele and Krone, 1996; for recent reviews, see Jowett, 1999, Meng *et al.*, 1999), although the technique was still poorly developed. In order to determine critical life stages and sensitive target tissues in (xeno-)estrogen responsive transgenic zebrafish, luciferase reporter gene induction was studied in various stages of development as well as in tissues of adult zebrafish following short term (xeno-)estrogenic exposure (Chapter 5). In addition, the expression of ER α and β mRNA during developmental stages and in various tissues was studied and related to luciferase induction (Chapter 5).

In Chapter 6, both the ER-CALUX assay and transgenic zebrafish assay were applied to determine estrogenic potency in WTP effluents, as well as a small panel of (xeno-) estrogens. The differences in response to (mixtures of) (xeno-)estrogens between the *in vitro* and *in vivo* assay were elucidated and discussed (Chapter 6).

In Chapter 7, the perspectives for the use of the newly developed bioassays in the field of environmental toxicology are discussed. The possible impacts of current levels of (xeno-)estrogens in the Dutch aquatic environment as measured with the newly developed bioassays are also discussed.

Chapter 2

Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line

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Abstract

Development of an estrogen receptor-mediated chemical activated luciferase reporter gene expression (ER-CALUX) assay was attempted by stable transfection of luciferase reporter genes in a number of cell lines. Stable transfection of the chimeric Gal4 estrogen receptor and luciferase gene constructs in MCF-7 breast cancer and Hepa.1c1c7 mouse hepatoma cell lines, as well as transfection of a newly constructed luciferase reporter gene pEREtata-Luc in the ECC-1 human endometrial cell line, resulted in constitutive, non-estradiol inducible clones. Stable transfection of pEREtata-Luc in the T47D breast cancer cell line, however, resulted in an extremely sensitive, highly responsive cell line. Following 24 hour exposure to estradiol (E2), stably transfected T47D.Luc cells demonstrated a detection limit of 0.5 pM, an EC50 of 6 pM and a maximum induction of 100-fold relative to solvent controls. No clear reduction in responsiveness has been found over extended culture periods (50 passages). Anti-estrogens ICI 182,780, TCDD and tamoxifen inhibited the estradiol-mediated luciferase induction. Genistein, nonylphenol and o,p'DDT were the most potent (pseudo-)estrogens tested in this system (EC50 100, 260 and 620 nM, resp.). Determination of interactive effects of the (pseudo-)estrogens nonylphenol, o,p'DDT, chlordane, endosulfan, dieldrin and methoxychlor revealed that, in combination with 3 pM E2, pseudo-estrogens were additive. Slightly more than additive effects (less than 2-fold) were found for combinations of dieldrin and endosulfan tested in the range of 3 to 6 μM. At these concentrations, the combination of endosulfan and chlordane demonstrated additive interaction. The ER-CALUX assay with T47D cells can provide a sensitive, responsive and rapid *in vitro* system to detect and measure substances with potential (anti-)estrogenic activity.

Introduction

An increasing number of reports that chemicals in the environment may mimic the natural hormone estrogen, thereby disrupting normal endocrine function, have raised public concern and resulted in a rapidly growing research effort. It has been hypothesized that increased incidence of hormone-sensitive cancers, decreased sperm counts in humans and compromised gonadal functionality in wildlife may be due to exposure to substances possessing estrogenic activity (reviewed by Colborn *et al.*, 1993). An ever-growing list of environmental, industrial, natural and pharmaceutical chemicals have been identified as potentially estrogenic. Many of these chemicals, defined as "estrogenic", have structures that deviate considerably from the natural steroid hormone 17β-estradiol, but can nevertheless evoke effects via the estrogen receptor signal transduction pathway. The effects of estrogens are mediated via binding with high affinity to a specific estrogen receptor (ER), a member of the nuclear receptor superfamily (Evans, 1988). In addition to the "classical" ERα form, recently a second ER form (ERβ) has been identified that has high homology with ERα but shows a different tissue distribution, ligand binding potential and trancriptional activity (Mosselman *et al.*, 1996; Kuiper *et al.*, 1996; 1997; 1998). Estrogen freely enters the cell and binds to the ER, inducing heat shock protein 90 dissociation and homodimerization (Figure 1). The homodimer complex binds to specific DNA sequences called estrogen response elements (ERE) within regulatory

regions of estrogen-responsive genes. Transcription is induced and messenger RNA is translated into protein. The synthesis of these new proteins can alter cellular functions and result in profound effects on physiology (Ing and O'Malley, 1995). In females, estrogen is essential for the development of secondary sex characteristics, the reproductive cycle, fertility and maintenance of pregnancy. Recently, estrogens have been shown to be essential for male fertility as well (Hess *et al.*, 1997).

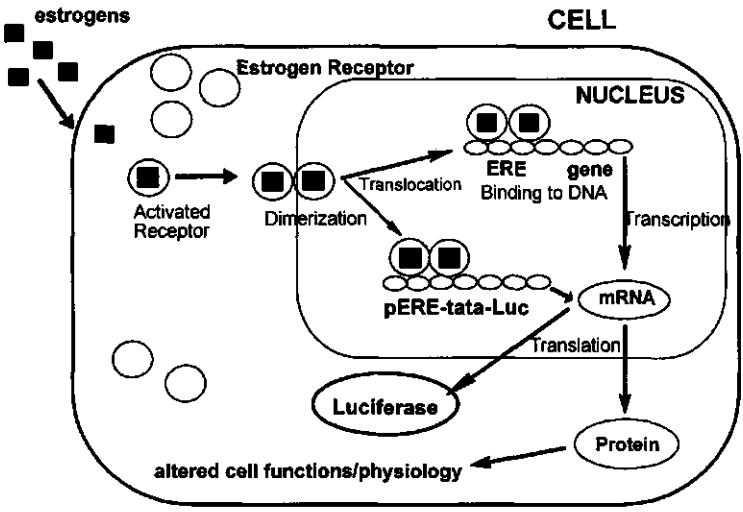

Figure 1: Mechanism of estrogen action in stably transfected T47D.Luc cells. (Pseudo-) estrogens enter the cell and bind to and activate the estrogen receptor (ER). Following dimerization, the ER-ligand dimer translocates to the cell nucleus and binds to estrogen response elements (ERE) upstream of genes or present on pEREtata-Luc construct. Transcription and translation of proteins (including luciferase) is induced.

A number of *in vitro* assays have been developed to screen substances for (anti-)estrogenicity (see review by Zacharewski, 1997). These assays include competitive ligand binding assays (Korach *et al.*, 1978; Berthois *et al.*, 1986; Migliaccio *et al.*, 1992), cell proliferation assays (Soto *et al.*, 1995), recombinant receptor/reporter gene assays (Pons *et al.*, 1990; Jausons-Loffreda *et al.*, 1994) and yeast-based screens for estrogens (Connor *et al.*, 1995; Arnold *et al.*, 1996; Routledge and Sumpter, 1997). However, most of these assays suffer from a number of drawbacks that limit their usefulness as a screen for (anti-)estrogenicity. Competitive ligand binding assays for the estrogen receptor are limited because they cannot distinguish between estrogenic and anti-estrogenic substances and do not provide insight into a substance's ability to initiate the molecular cascade of events leading to altered gene expres-

sion. Cell proliferation assays are limited by a lack of specificity as mitogens other than estrogens are able to influence the proliferation of human breast cancer cells (van der Burg *et al.*, 1988; Dickson and Lippman, 1995). Yeast-based assays are simple and sensitive, however drawbacks include lack of responsiveness to anti-estrogens (Lyttle *et al.*, 1992; Kohno *et al.*, 1994) as well as possible differences in permeability of compounds through the yeast cell wall relative to mammalian cell membranes.

In comparison to other existing *in vitro* assays, we believe recombinant receptor and reporter gene assays based on stably transfected cell lines can provide one of the most specific, responsive and biologically relevant means to screen substances for potential anti-estrogenic as well as estrogenic effects with a high throughput potential. This type of assay is based on the receptor-mediated mechanism of action of estrogens and reporter gene expression is a culmination of molecular cascade of events involved in receptor transactivation. To our knowledge, only two estrogen-responsive cell lines stably transfected with recombinant receptor and/or reporter genes have previously been developed. Pons and coworkers developed a MCF-7 cell line stably transfected with the pVit-tk-Luc construct which consists of the *Xenopus laevis* vitellogenin A2 promoter region containing one estrogen response element upstream of the *Herpes simplex* virus thymidine kinase promoter controlling expression of the firefly luciferase gene (Pons *et al*, 1990). A HeLa cell line stably transfected with a Gal4-regulated chimeric estrogen receptor and luciferase reporter gene constructs has also been developed (Jausons-Loffreda *et al.*, 1994). In both stably transfected cell lines, however, only modest sensitivity and responsiveness to estradiol has been reported.

Recently in our laboratories, stably transfected CALUX (chemical activated luciferase gene expression) cell line assays have been developed as a sensitive and rapid method to determine the potency of individual congeners and mixtures of dioxin-like compounds (Aarts *et al.*, 1995; Garrison *et al.*, 1996; Murk *et al.*, 1996). These cell lines express endogenous aryl hydrocarbon (Ah) receptor and are stably transfected with a dioxin-responsive luciferase reporter gene. In the present study we attempted to develop new, highly responsive stably transfected estrogen receptor-mediated ER-CALUX cell lines for the assessment of (anti-)estrogenic substances. ECC-1 human endometrial carcinoma and T47D human breast adenocarcinoma cells expressing endogenous estrogen receptor were stably transfected with a newly constructed estrogen-responsive luciferase reporter gene, pEREtata-Luc. This construct consists of three estrogen response elements upstream of a TATA box regulating expression of an enhanced luciferase reporter gene construct. Hepa.1c1c7 mouse hepatoma and MCF-7 human breast adenocarcinoma cells were stably transfected with the chimeric receptor and reporter gene constructs Gal4-HEGO and 17m5-G-Luc. Stable transfection of pEREtata-Luc in T47D cells resulted in a sensitive, highly responsive clone which was further characterized in dose-response studies with estradiol as well as some known estrogenic and anti-estrogenic substances.

Materials and Methods

Chemicals

17β-estradiol (E2, 99%), methoxychlor (95%), tamoxifen (99%), genistein (99%), al-trans retinoic acid and ethanol (100%, p.a.) were purchased from Sigma Chemicals Co. 4-nonylphenol (92.7%) was purchased from Fluka. Bisphenol A (99%) was purchased from Aldrich. Chlordane (97.7%), endosulfan (99%) and dieldrin (98.5%) were purchased from Riedel-de Haan, The Netherlands. ICI 182, 780 was a kind gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, U.K. o,p'DDT and tetrachlorodibenzo-p-dioxin (TCDD) were kindly provided by the Dutch State Institute for Quality Control of Agricultural Products (RIKILT-DLO). The synthetic progestin Org 2058 was a gift from Organon B.V., Oss, The Netherlands. The synthetic androgen R1881 (methyltrienolone) was purchased from NEN (Boston, U.S.A.) and kindly provided by Dr. A. Brinkman, Erasmus University, Rotterdam. For exposure of cells, all chemicals were dissolved in ethanol or dimethyl sulfoxide (DMSO, 99.9%, spectrophotometric grade, Acros). Antibiotics used for selection of stable clones (puromycin, hygromycin and G418) were purchased from Duchefa, The Netherlands.

Cell culture

The T47D human breast adenocarcinoma cell line was kindly provided by Dr. R.L. Sutherland (Garvan Institute of Medical Research, Sydney, Australia). The T47D cells were cultured in a 1:1 mixture of Dulbeccos's modified Eagle's medium and Ham's F12 medium (DF, Gibco) supplemented with sodium bicarbonate, non-essential amino acids, sodium pyruvate and 7.5% fetal calf serum (FCS, Integro, Austria). T47D cells were cultured at 37°C, 7.5% CO_2. The ECC-1 human endometrial carcinoma cell line was a kind gift from Dr. P.G. Stayaswaroop (Pennsylvania State University, U.S.A). ECC-1 cells were cultured in RPMI (Gibco) supplemented with 1 ng/ml insulin and 5% FCS (Sigma). The MCF-7 human breast cancer cell line was acquired from the American Type Culture Collection (ATCC, USA). The Hepa 1c1c7 mouse hepatoma cell line was kindly provided by Dr. J.P. Whitlock, Jr. (Stanford University, U.S.A.) The MCF-7 and Hepa cell lines were cultured according to Zacharewski *et al.*, 1995 in phenol red-free Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 10% FCS. ECC-1, MCF-7 and Hepa cells were cultured at 37°C, 5% CO_2.

For ER-CALUX assays, T47D, MCF-7 and Hepa cells were maintained in assay medium without phenol red supplemented with 5% dextran-coated charcoal treated FCS (DCC-FCS). DCC-FCS was prepared by heat inactivation (30 minutes at 56°C) of FCS followed by two 45-minute DCC treatments at 45°C as described by Horwitz and McGuire, 1978. ECC-1 cells were assayed in RPMI with phenol red supplemented with 5% DCC-FCS.

Receptor and reporter gene constructs

The reporter gene pEREtata-Luc was constructed using the enhanced luciferase reporter gene pGL3-basic (Promega, U.S.A.). Three tandem repeats of the consensus ERE oligo (GAGCTTAGGTCACTGTGACCT) upstream of the minimal human E_1B TATA promoter sequence (GGGTATATAAT) were inserted in the Sma1-Bgl II site of the multiple cloning site of pGL3-basic (Figure 2).

The Gal4-HEGO chimeric receptor and Gal4-regulated luciferase reporter gene 17m5-G-Luc were kindly provided by Prof. P. Chambon, INSERM U184, Strasbourg, France. Gal4-HEGO consists of the ligand binding domain of the ER linked to the DNA binding domain (1-147) of the yeast transcription factor Gal4 (Green *et al.*, 1988). The plasmid 17m5-G-Luc consists of luciferase cDNA regulated by the rabbit β-globin basal promoter and five tandem consensus Gal4 17-mer response elements (Jausons-Loffreda *et al.*, 1994). For stable transfection, a second 17m5 luciferase reporter gene (p17m5-G-neo-Luc) was constructed by cloning the Pvu1-EcoRV fragment of pMAMneo-Luc (ClonTech, U.S.A.) containing the sequence for neomycin (G418) resistance in the Pvu1- EcoRV sites of p17m5-G-Luc.

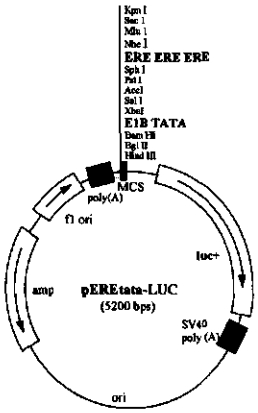

Figure 2: Schematic representation of the pEREtata-Luc construct. Three estrogen response elements (ERE) upstream of a minimal E_1B TATA promoter were inserted in the multiple cloning site (MCS) of the enhanced luciferase (luc+) vector pGL3-basic.

For selection of stable transfected clones, the following selection plasmids were used: for puromycin selection, pPur (ClonTech, U.S.A.); for hygromycin selection. pGK-Hyg (Te Riele *et al.*, 1990); and for G418 selection, pSV2-Neo (Hoglund *et al.*, 1992).

Stable transfection

ECC-1, MCF-7 and Hepa.1c1c7 cell lines

Six-sixteen hours prior to transfection, ECC-1, MCF-7 and Hepa.1c1c7 cells were seeded at 50% confluency in three wells of a 6-well plate in 3 ml culture medium per well. Transfection was carried out according to the calcium phosphate precipitation method (Sambrook *et al.*, 1989). In ECC-1 cells, 4.65 mg pEREtata-Luc and 4.65 mg pPur was

transfected per well, using 250 ml Hepes-buffered solution (HBS, consisting of 42 mM Hepes, 275 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, pH 7.05) and 250 ml 250 mM $CaCl_2$ per well. For MCF-7 and Hepa cells, cells were transfected with either 0.6 mg pGal4HEGO, 3 mg p17m5-G-Luc and 1.5 mg pSV2Neo or 0.6 mg pGal4HEGO and 3 mg p17m5-G-neo-Luc. After 24 hours, the cells were trypsinized and per well distributed over five 100 mm petri dishes. Medium was replaced every three days with culture medium containing 1 mg/ml puromycin (ECC-1 cells) or 1000 mg/ml G418 (MCF-7 and Hepa cells) until clones formed which were large enough to isolate (about two to three weeks).

Luciferase positive clones were screened using a single photon detecting camera (Argus 50, Hatamatsu) according to the method described by Pons *et al.*, 1990. Briefly, clones in 100 mm dishes were exposed for 24 hours to 10 nM E2. Luciferin (0.3 mM) was added to the culture medium and dishes were immediately introduced into the dark room of the single-photon counting camera for luminescence measurement. Light intensity was monitored for 5 to 10 minutes per dish. The image of the luminescent cells was superimposed on to the light field image of the dishes, enabling identification of positive clones. Positive clones were isolated using cloning rings and further cultured in 24 well plates.

To test for luciferase induction, clones were trypsinized and seeded over 3 wells in a white 96-well viewplate (Packard) in assay medium. Following 24 hour incubation, cells were exposed for 24 hours to control (0.5% DMSO), 10 nM E2 and 10 nM ICI 182,780. Cells were rinsed twice with 50 ml 0.5x phosphate buffered saline (PBS). Low salt (LS) buffer containing 10 mM Tris pH 7.8, 2 mM DTT, 2 mM 1,2 diaminocyclohexane-N,N,N',N'-tetraacetic acid was added at a volume of 20 ml per well. Following 10 minute incubation on ice, 96 well plates were frozen at -80°C for a minimum of 30 minutes to lyse cells. The plates were thawed on ice and shaken for 5 minutes at room temperature. Luciferase activity was measured in 96 well viewplates in a luminometer (Labsystems Luminoscan RS) with automatic injection of 100 ml luciferin "flashmix" containing 470 mM luciferin, 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)2.5H_20$, 2.67 $MgSO_4$, 0.1 mM EDTA, 5 mM ATP, 2 mM DTT, pH 7.8 per well.

pEREtata-Luc in T47D cell line

Two days prior to transfection, T47D cells were plated at a density of 250 000 cells in 5 cm petridishes in 5 ml culture medium. Per dish, 18 mg pEREtata-Luc and 2 mg pGK-Hyg was transfected using the calcium phosphate precipitation method described above. Cells were incubated with transfection mixture for 8 hours, after which the medium was renewed. The following day, the cells were trypsinized and plated over four 5 cm dishes per dish in culture medium supplemented with 100 mg/ml hygromycin (selection medium). Clones were allowed to grow for 10 days, during which time the selection medium was renewed every two to three days. Dishes containing 10-15 large colonies were trypsinized for 1-3 minutes at room temperature. Individual clones were resuspended in 2 ml medium and added to 24 well plates containing 2 ml selection medium. Smaller clones were left an extra 2 weeks to grow and isolated in the same manner. To test for luciferase induction, confluent 24 wells were trypsinized and one 96 well per clone was seeded in culture medium and incubated for 24 hours. Non-transfected T47D cells were used as control. Medium was removed and cells were

lysed in 55 ml triton-lysis buffer containing 1% triton X-100, 25 mM glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA (pH 7.8) and 1 mM DTT. A 50 ml portion of cell lysate was transferred to a black 96 well plate (Packard) to which 50 ml luciferine substrate (LucLite reporter gene assay kit, Packard) was added. Luciferase activity was measured in a scintillation counter (Packard Top Count) for 0.1 minute per well.

ER-CALUX assay procedure

T47D cells stably transfected with pEREtata-Luc from the most responsive clone were plated in 24 well plates at a density of 50 000 cells in 0.8 ml DF without phenol red + 5% DCC-FCS (assay medium) per well or in black 96 well viewplates (Packard) at a density of 5000 cells per well in 0.1 ml assay medium. Following 24 hours incubation, cells were approximately 50% confluent. Assay medium was renewed and the cells were incubated another 24 hours. The medium was then renewed again and the cells were dosed in triplicate in 24 well plates by direct addition of the chemical to be tested dissolved in ethanol or DMSO to the medium above cells. For 96 wells plates, assay medium containing chemicals was first prepared in 0.8 ml assay medium in 48 well plates, mixed well, and transferred to the microtiter plate at a volume of 0.1 ml per well. In addition to one E2 standard curve in triplicate per experiment, control wells, solvent control wells and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. The maximum solvent concentration used was 0.2%. Cells were dosed for 24 hours prior to luciferase measurement. For 24 well plates, medium was removed, and the cells were lysed in 100 ml triton-lysis buffer. Lysis was carried out by gentle shaking at 4°C for a minimum of one hour. A 50 ml sample of the cell lysate was then transferred to a black 96-well plate, 50 ml luciferin substrate was added and the luciferase activity was assayed in a scintillation counter for 0.1 minute per well. For 96 well viewplates, 50 ml LucLite was added directly to the medium above cells and the plates were shaken gently for 10 minutes at room temperature to stimulate cell lysis. The transparent bottom of the viewplates was covered by a black sticker prior to luciferase measurement in a scintillation counter (Packard Top Count).

Potential cytotoxicity of pseudo-estrogens was controlled by microscopic visualization of the cells. In addition, a "CytoLite" (Packard) luminescent, non-separation assay kit for the determination of viable cell numbers was used according to manufacturer's specifications in black 96-well viewplates. T47D.Luc cells were seeded and exposed to pseudo-estrogens in the same manner as outlined in the ER-CALUX assay procedure.

Data and statistical analysis

To determine the EC50 and detection limit of E2 in the ER-CALUX assay, a complete standard curve was included in each assay. The standard curve was fitted (sigmoidal fit, function: $y=a_0+a_1/(1+\exp(-(x-a_2)/a_3))$ using SlideWrite 3.0 for Windows, which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion). The EC50 for E2 and pseudo-estrogens was calculated by determining the concentration at which 50% of the maximum luciferase activity was reached using the sigmoidal fit equation. The detection limit was calculated as the luciferase activity elicited by the solvent control plus three times the standard deviation.

In order to determine estradiol equivalents (EEQs), the luciferase response by (combinations of) pseudo-estrogens was interpolated in the linear range of the corresponding E2 standard curve for the same assay. Data shown are representative of a minimum of two independent assays. Statistical analysis was performed on luciferase activity by combinations of pseudo-estrogens as compared to the arithmetic sum of luciferase activity by individual compounds using ANOVA (α=0.01) in SPSS Version 6.0 for Windows. Standard deviation around the arithmetic sum was calculated by taking the square root of the pooled variance of the luciferase activity by the two individual compounds.

Results

Stable transfection of reporter genes in ECC-1, MCF-7 and Hepa cell lines

Following transfection of the pEREtata-Luc reporter gene plasmid in ECC-1 cells and selection with puromycin, 23 luminescent clones were identified using the single photon counting camera. However, exposure of these clones to 10 nM E2 resulted in no inducible luciferase activity. Luciferase expression in the clones appeared to be constitutive. Similar results were obtained with Hepa.1c1c7 cells transfected with the chimeric receptor and reporter gene constructs pGal4-HEGO and p17m5-G-Luc and selected with G418. Fifteen luminescent clones were identified with the photon-counting camera, of which only one demonstrated estradiol-inducible luciferase expression. This clone showed a 5-fold increase in luciferase activity relative to background following 24 hour exposure to 10 nM E2. In subsequent assays, however, luciferase activity decreased to three times control levels, suggesting that integration was unstable (data not shown). In Hepa cells transfected with pGal-HEGO and p17m5-G-neo-Luc, all 12 luminescent clones showed constitutive, estradiol-independent luciferase expression. Three separate attempts to stably transfect the chimeric constructs in MCF-7 cells with different concentrations of G418 selection resulted in the formation of unstable clones which ultimately died.

Stable transfection of pEREtata-Luc in T47D cells

A total of 72 T47D clones were isolated and tested for luciferase expression by comparison of activity (without estradiol stimulation) relative to non-transfected cells. Using this method, 28 clones were identified with luciferase expression more than 10-fold induction relative to non-transfected cells. Of these 28 clones, 22 were further characterized according to the ER-CALUX assay method in 24 well plates with exposure to 1 nM estradiol. Nine clones were found with greater than 10-fold induction relative to control. Clone ERE.Luc number 42 showed the highest induction and was used for further experiments with the ER-CALUX method.

ER-CALUX assay with stably transfected T47D cells

Stable transfection of the pEREtata-Luc contruct in T47D cells resulted in a sensitive, highly responsive cell line for the assessment of compounds with (anti-)estrogenic activity. As illustrated in Figure 1, using the ER-CALUX assay with T47D.Luc cells, substances can be tested for (anti-)estrogenicity based on their specific estrogen receptor mediated mecha-

nism of action. Upon entering the T47D.Luc cell, substances with estrogenic activity will bind to the endogenous estrogen receptor and activate the receptor. The activated receptor dimer subsequently binds to estrogen response elements (EREs) upstream of the luciferase gene, leading to recruitment of transcription factors to the promoter and ultimately to luciferase gene expression (Figure 1).

Response to estradiol

The sensitivity and responsiveness of the ER-CALUX assay with the stably transfected T47D ERE.Luc 42 clone (T47D.Luc cells) was assessed by measuring the luciferase activity induced by E2 compared to solvent control over a period of 9 months in cells ranging from passage 9 to 48. Exposure of T47D.Luc cells to estradiol for 24 hours resulted in dose-response related luciferase induction as measured in both 24 well plates and 96 well plates (Figure 3). In 24 well plates, averaged over 15 assays, 102 (±76) fold maximum induction relative to control was reached at 30 pM E2. A detection limit of 0.5 (±0.1) pM and an EC50 of 5.5 (±1.8) pM was calculated. Using 96 wells plates, a similar EC50 and detection limit was measured in 6 experiments, though maximum induction at 30 pM E2 relative to control was less than in 24 well plates (average 76 (± 31) fold). No clear reduction in luciferase activity over prolonged periods of cell culture was found, demonstrating stable integration of the luciferase gene.

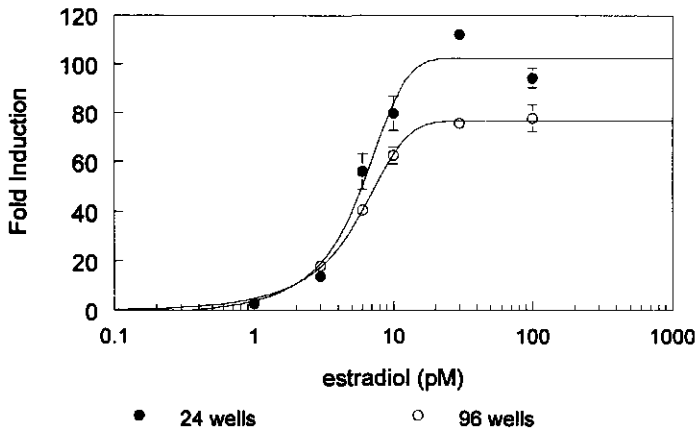

Figure 3: Luciferase induction by estradiol relative to solvent control in the ER-CALUX assay with T47D cells stably transfected with pEREtata-Luc plated in 24 (solid circles) or 96 (open circles) well plates. Cells were treated with estradiol for 24 hours (n=3, avg ± std).

Response to other steroids and retinoic acid

Because the T47D cell line expresses other hormone receptors, such as progesterone, androgen and retinoic acid receptors, we determined the potential effects of other endogenous ligands on the estrogen receptor mediated luciferase response. Luciferase activity in T47D.Luc cells was determined following 24 hour treatment with a synthetic progestin (Org 2058), synthetic androgen (R1881) and retinoic acid. No luciferase activity was found by these ligands when tested in concentrations ranging from 1 pM to 10 nM (data not shown).

Response to anti-estrogens

Exposure of T47D.Luc cells to the pure anti-estrogen ICI 182,780 and the Ah receptor agonist TCDD at concentrations ranging from 0.01 to 1000 nM resulted in luciferase activity equal to or slightly less than control values (Figure 4). When tested in combination with the EC50 value (6 pM E2), ICI 182,780 completely inhibited luciferase activity at concentrations at or above 1 nM. TCDD was a potent antagonist when co-administered with 6 pM E2, as a concentration as low as 10 pM inhibited luciferase activity by 50% (Figure 4). Exposure of cells to tamoxifen alone at lower concentrations (< 100 nM) resulted in no luciferase activity, though tamoxifen was slightly estrogenic at 100 and 1000 nM (about 10% activity relative to 6 pM E2) (Figure 4). Tamoxifen was far less anti-estrogenic when tested in combination with E2 than ICI 182, 780, as concentrations above 100 nM were necessary to inhibit the E2-induced luciferase response by about 90%.

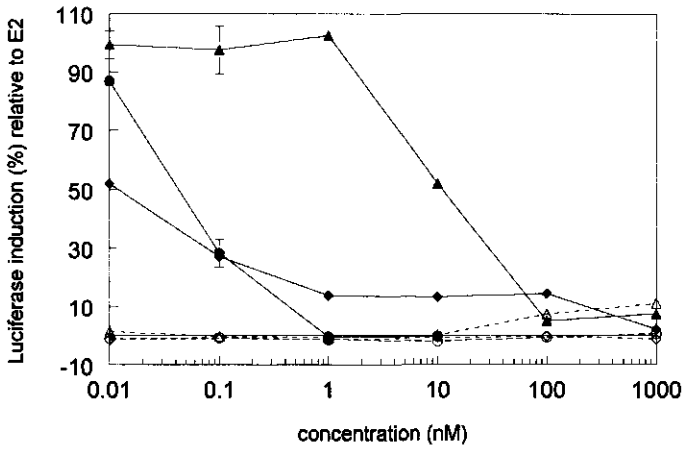

Figure 4: Luciferase activity (%) of anti-estrogens relative to estradiol (E2, 6 pM) in the ER-CALUX assay. T47D.Luc cells were treated for 24 hours with tamoxifen (triangles), ICI 182,780 (circles) and TCDD (diamonds). Anti-estrogens were tested with (solid lines) or without (dashed lines) co-administration of 6 pM E2 (n=3, avg ± std).

Response to pseudo-estrogenic compounds

The specificity of the ER-CALUX assay was determined by testing the luciferase activity induced by a number of compounds previously shown in other in vitro assays to be estrogenic. These compounds were also estrogenic when tested in T47D.Luc cells (Table 1). Genistein, nonylphenol and o,p'DDT were the most potent pseudo-estrogens tested (EC50 100, 260 and 660 nM, resp.) (Table 1). With the exception of dieldrin and methoxychlor, all compounds tested at concentrations above 30 μM demonstrated cytotoxic effects (data not shown). The organochlorine pesticides tested induced luciferase in a dose-response related manner (Figure 5). A number of compounds, including nonylphenol, genistein, o,p'DDT, methoxychlor and bisphenol A, showed maximum luciferase activity exceeding that of estradiol at 30 pM, though at concentrations 3000 - 300000 times that of estradiol (Table 1).

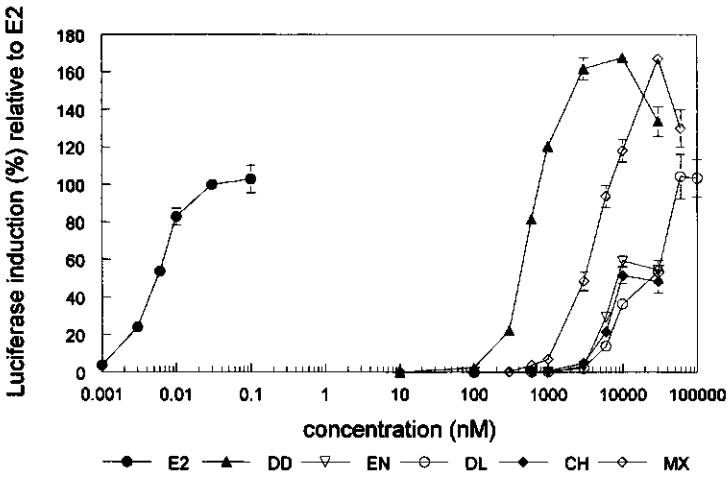

Figure 5: Luciferase induction (%) of pesticides relative to 30 pM estradiol (E2) in the ER-CALUX assay. T47D.Luc cells were treated for 24 hours with o,p'DDT (DD), chlordane (CH), endosulfan (EN), dieldrin (DL) and methoxychlor (MX) (n=3, avg ± std).

Combination effects of pseudo-estrogens

Pseudo-estrogenic compounds were tested in combination with a low concentration of E2 (3 pM) in order to determine potential interactive effects when tested in mixtures (Figure 6). All combinations tested showed additive luciferase activity, i.e. not significantly different than the arithmetic sum of the compounds tested individually. To determine interactive effects, the pseudo-estrogens endosulfan, dieldrin and chlordane were also tested together at concentrations eliciting luciferase activity within the lower linear range of the estradiol

Table 1: Potency of (pseudo-)estrogens relative to estradiol following 24 hour treatment in the ER-CALUX assay with T47D.Luc cells

	LOEC[a] (nM)	Relative potency[b] (LOEC)	EC50[c] (nM)	Relative potency[d] (EC50)	Max. effect conc. (nM)[e]	% effect rel. to estradiol[f]
Natural hormone:						
Estradiol	0.0005		0.006	1	0.030	100
Pesticides:						
o,p'-DDT	100	5 E-6	660	9.1 E-6	10000	168 (2.0)
Methoxychlor	600	8 E-7	5720	1.0 E-6	30000	167 (12.9)
Dieldrin	1000	5 E-7	24490	2.4 E-7	60000	104 (11.8)
Endosulfan	1000	5 E-7	5920	1.0 E-6	10000	59 (2.6)
Chlordane	1000	5 E-7	6240	9.6 E-7	10000	52 (4.5)
Industrial chemicals:						
4-nonylphenol	100	5 E-6	260	2.3 E-5	1000	135 (1.3)
bisphenol A	100	5 E-6	770	7.8 E-6	10000	153 (2.3)
Phytoestrogen:						
genistein	100	5 E-6	100	6.0 E-5	1000	127 (2.4)

[a] low effect concentration; test concentration at which estrogenic effect is detected
[b] ratio between dose of compound and that of estradiol needed to achieve an estrogenic effect
[c] concentration at which 50% of maximum luciferase activity is reached
[d] ratio between EC50 of compound and EC50 of estradiol
[e] concentration at which maximum estrogenic effect is detected
[f] Per cent luciferase activity (standard deviation) at concentration eliciting maximum estrogenic effect relative to 30 pM estradiol

standard curve (between 1 and 6 pM E2 equivalents). The combination of endosulfan with dieldrin at concentrations between 3 and 6 μM resulted in luciferase activity showing more than additive interaction. The luciferase activity elicited by these combinations significantly exceeded the arithmethic sum of their individual luciferase activity, though by a factor of less than 2 (Figure 7). At 7 and 8μM, the interaction between endosulfan and dieldrin was additive or slightly less than additive (Figure 7). The combination of endosulfan and chlordane tested at concentrations between 3 and 5μM resulted in additive luciferase activity (Figure 8). When combined at concentrations higher than their individual EC50 values (6 to 8 μM), luciferase activity was less than additive (Figure 8).

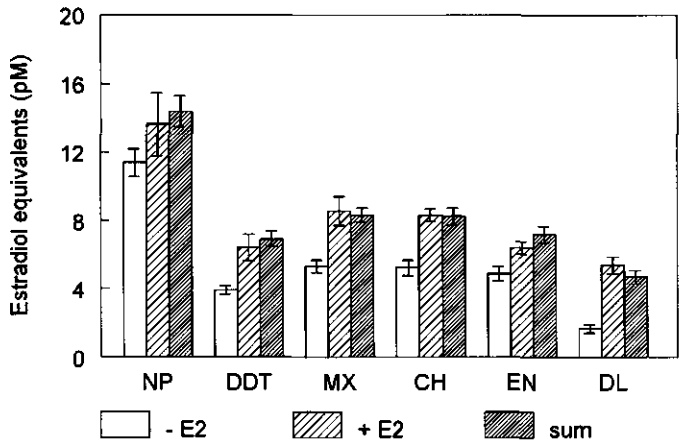

Figure 6: Interactive estrogenic activity of environmental chemicals with estradiol (E2) in the ER-CALUX assay. T47D.Luc cells were exposed for 24 hours to environmental chemicals with (striped bars) or without (open bars) co-administration of 3 pM E2. Double-striped bars show the arithmetic sum of luciferase activity obtained by adding the induction by the individual compound with induction elicited by 3 pM E2. o,p'DDT (DDT) and 4-nonylphenol (NP) were tested at a concentration of 200 nM. Methoxychlor (MX) was tested at a concentration of 2 μM. Chlordane (CH), dieldrin (DL) and endosulfan (EN) were tested at a concentration of 4 μM (n=6, avg ± std).

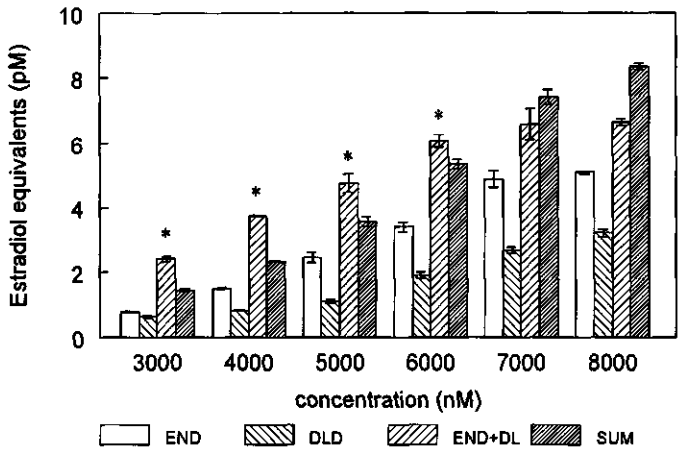

Figure 7: Interactive effects of mixtures of the pseudo-estrogens endosulfan (END) and dieldrin (DLD) in the ER-CALUX assay. T47D.Luc cells were treated with individual and combinations (COMB) of environmental estrogens. Double-striped bars on the right show the arithmetic sum (SUM) of luciferase activity obtained by adding the induction elicited by the individual compounds. (n=6, avg ± std, *significantly higher than arithmetic sum, $p<0.01$).

Discussion

Stable transfection in ECC-1, MCF-7 and Hepa cell lines

Attempts to stably transfect the pEREtata-Luc construct in ECC-1 cells, as well as the chimeric Gal4-HEGO/17m5-G-Luc constructs in Hepa.1c1c7 and MCF-7 cells resulted in constitutive, non-estradiol-inducible luciferase expression. Jausons-Loffreda and co-workers also reported some hormone-independent luminescent clones after stable tranfection of chimeric Gal4 constructs in HeLa cells (Jausons-Loffreda *et al.*, 1994). The reasons for these constitutive clones are not clear, but may be due to events during integration resulting in a mutation in the promoter region of the luciferase gene, or influence of promoters around the integration site, causing constitutive, E2-independent luciferase expression. Only one Hepa clone stably transfected with Gal4HEGO/17m5-G-Luc demonstrated estradiol-inducible luciferase activity, though this induction decreased in subsequent assays, indicating instability of the clone. Subculturing of this clone could result in clones with a more homogeneous population of luciferase-expressing cells.

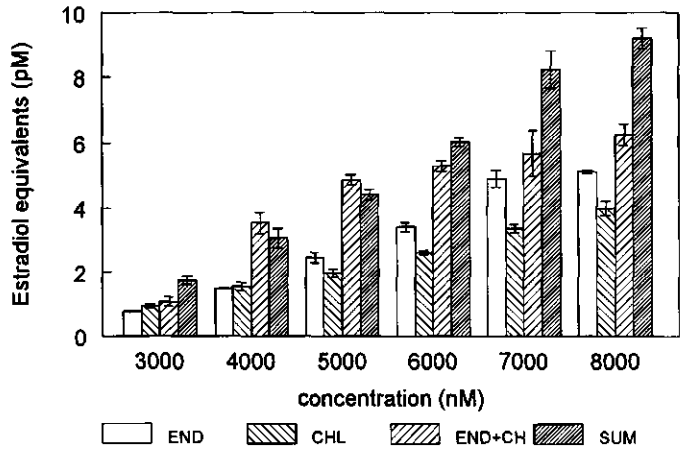

Figure 8: Interactive effects of mixtures of the pseudo-estrogens endosulfan (END) and chlordane (CHL) in the ER-CALUX assay. T47D.Luc cells were treated with individual and combinations (COMB) of environmental estrogens. Double-striped bars on the right show the arithmetic sum (SUM) of luciferase activity obtained by adding the induction elicited by the individual compounds (n=6, avg ± std, EC50 endosulfan: 5920 nM; EC50 chlordane: 6240 nM).

ER-CALUX assay in stably transfected T47D cell line

Stable transfection of the pEREtataLuc construct in T47D cells resulted in a sensitive, highly responsive cell line for the screening of compounds with (anti-)estrogenic activity. Estradiol concentrations as low as 0.5 pM were detected and an EC50 of 6 pM was found following 24 hour exposure to estradiol. The assay is also highly responsive, as luciferase induction in 24 well plates by 30 pM E2 resulted in about 100 fold relative to solvent controls. The assay appears also to be specific for estrogens, as other endogenous ligands tested, such as a synthetic progestin and androgen, and retinoic acid, did not induce luciferase activity. Anti-estrogenicity of substances such as ICI 182,780 and tamoxifen could be demonstrated by measuring inhibition of luciferase induction with co-administration of estradiol. The prototypical Ah receptor agonist TCDD was also a potent anti-estrogen when measured in the ER-CALUX assay. These results are consistent with previous studies which demonstrate the anti-estrogenicity of TCDD in breast cancer cell lines, likely due to the complex cross talk between the AhR and ER signal transduction pathways (reviewed by Safe, 1995).

Response of pseudo-estrogens

A number of (pseudo-)estrogenic compounds were tested individually and in combination in order to determine their potency in the ER-CALUX assay. The phyto-estrogen genistein, nonylphenol and the pesticide o,p'DDT were the most potent of the pseudo-estrogens tested. The environmental estrogens demonstrating induced luciferase activity in the ER-CALUX assays have also been previously shown to be estrogenic in both *in vivo* and *in vitro* assays: 4-nonylphenol (Soto *et al*, 1991; Jobling and Sumpter, 1993; White et al., 1994), o.p'DDT (Bitman, 1969, Fry and Toone, 1981, Klotz et al., 1996), methoxychlor (Gray *et al.*, 1989, Schlenk *et al.*, 1997), endosulfan (Arnold *et al.*, 1996), dieldrin (Haake *et al.*, 1987) and chlordane (Cranmer *et al.*, 1984). Comparison of the potency values relative to estradiol shows that in general, lower potency values for environmental estrogens are calculated in the ER-CALUX than demonstrated with other ER-mediated reporter gene assays, such as transient transfections of ERE-reporter genes in HeLa and MCF-7 cells (Table 2). However, this factor 10-100 fold difference in potency could be explained by the differences in estradiol concentrations required to produce a maximal response. For the ER-CALUX assay, maximum luciferase induction is reached at 30 pM E2. For other assays, estradiol concentrations ranging from 1 nM (Shelby *et al.*, 1996) to 10 nM (White *et al.*, 1994) are used for calculation of relative potency. ER-CALUX relative potency values correlate well with those reported in MCF-7 cell proliferation assays, in which a maximum E2 effect concentration of 10-30 pM is also found (Soto *et al.*, 1992; 1994; 1995).

Interestingly, when tested at high concentrations (> 1000 nM), a number of compounds such as genistein, nonylphenol, bisphenol A, o,p' DDT and methoxychlor elicited luciferase induction higher than that of the maximum induction by estradiol (Table 1). In a screening of estrogenic compounds using a recombinant yeast strain, Routledge and Sumpter also reported that exposure to both the genistein and the alkylphenol 4-*tert*-octylphenol resulted in higher reporter gene activity than by estradiol (Routledge and Sumpter, 1997). The mechanism of this super-agonism is not clear, but may be due to stimulated receptor and/or co-activation factor renewal by pseudo-estrogens, or effects on luciferase stability.

When tested in combination with 3 pM E2 in the ER-CALUX assay, all test compounds showed additive luciferase activity (Figure 6). However, when the pseudo-estrogens endosulfan, dieldrin and chlordane were tested in combination in the ER-CALUX assay, the resulting interaction was not consistently additive. The combination of dieldrin with endosulfan resulted in luciferase activity slightly more than additive, though significantly higher, than the arithmetic sum of the individual compounds (Figure 7). The combination of endosulfan and chlordane was additive at the lower concentrations tested (3 to 6 μM). Gaido and coworkers have also reported the additive effects of endosulfan and chlordane when tested in combination in a yeast estrogen screen (Gaido *et al.*, 1997). Less than additive luciferase induction by the combination of endosulfan and chlordane was observed at higher concentrations (7 and 8 μM) (Figure 8). However, these concentrations were higher than the EC50 level of the individual compounds, so it is not surprising that the arithmetic sum of their individual activities was higher than activity elicited by the combination. These results indicate that weak environmental estrogens may act cumulatively and further research is warranted in the *in vivo* effects of mixtures of pseudo-estrogens.

Table 2: Potency values for maximum effect concentrations of (pseudo-)estrogens relative to estradiol according to *in vitro* assays for estrogenicity

Compound	Assay	Relative Potency	Reference
o,p'DDT	ER-CALUX	3 E-6	
	transient transfection MCF-7	1 E-4	Klotz *et al.*, 1996
	E-SCREEN	1 E-6	Soto *et al.*, 1994
methoxychlor	ER-CALUX	1 E-6	
	transient transfection HeLa cells	0	Shelby *et al.*, 1996
dieldrin	ER-CALUX	5 E-7	
	E-SCREEN	1 E-6	Soto *et al.*, 1994
Endosulfan	ER-CALUX	3 E-6	
	transient transfection HeLa cells	0	Shelby *et al.*, 1996
	E-SCREEN	1 E-6	Soto *et al.*, 1994
4-nonyl-phenol	ER-CALUX	3 E-5	
	transient transfection HeLa cells	1 E-3	Shelby *et al.*, 1996
	transient transfection MCF-7	1 E-3	White *et al.*, 1994
	E-SCREEN	3 E-6	Soto *et al.*, 1992
genistein	ER-CALUX	3 E-5	
	E-SCREEN	1 E-4	Mayr *et al.*, 1992

Comparison ER-CALUX with other in vitro assays

To our knowledge, in comparison to other recombinant receptor and/or reporter gene assays using stably or transiently transfected mammalian cells, the ER-CALUX assay is the most sensitive and responsive stably transfected estrogen-responsive cell line in existence (Table 3). The sensitivity and specificity of the ER-CALUX assay may be explained in part by the use of a minimal E_1B promoter (TATA box only) in the pEREtata-Luc construct. Enhancer and promoter regions are absent in this minimal promoter. Other promoters used in response element-regulated reporter genes generally consist of several hundred base pairs of 5' flanking regions which may cause higher background activation levels (Cavailles *et al.*, 1988; 1989). Also, the vigorous serum stripping method used may contribute to the low background levels in the ER-CALUX assay. The presence of serum-borne estrogens can result in high constitutive reporter gene activity and can be demonstrated by assessing the ability of anti-estrogens to reduce background levels (Bondy and Zacharewski, 1993). In the ER-CALUX assay, ICI 182,780 at concentrations up to 100 nM did not significantly antagonize bacground luciferase activity, demonstrating that serum-borne estrogens were very low. The ER-CALUX assay procedure was also optimized to ensure the lowest possible background levels. Cells are plated 2 days prior to exposure with a medium renewal step on the second and third day. These medium renewal steps reduced background levels of serum-borne estrogens by a factor of 2 (data not shown).

The high responsiveness of the ER-CALUX assay may be due in part to the choice of the cell line. The T47D cell line appears to provide the cellular environment, such as high estrogen receptor levels and the availability of transcription factors, necessary for gene transcription. The presence of both ERα and ERβ subtypes in T47D cells has been confirmed using RT-PCR (Dotzlaw *et al.*, 1996). Estrogen receptor levels in the cytosol of T47D cells have been reported to be 67.6 ± 6.2 fmol/mg cytosol protein (Watanabe *et al.*, 1990). The use of an enhanced luciferase reporter gene which was modified for optimal transcriptional activity in transfected eukaryotic cells (Sherf and Wood, 1994) may also have contributed to higher induction values. Comparison of EREtata constructs with enhanced vs normal luciferase in transient transfections in the 293 human embryonic kidney cell line showed a factor 5 increase in fold induction (data not shown).

The use of the ER-CALUX assay in stably transfected T47D.Luc cells offers many advantages in comparison to other *in vitro* bioassays for assessing estrogenic substances, such as transient transfections of reporter gene constructs, yeast based assays and cell proliferation assays. The ER-CALUX assay is rapid and simple, and detection in microtiter volumes is possible, allowing for qualitative and quantitative assessment of luciferase induction in a high throughput setup. Compared to transient transfections of estrogen-responsive reporter genes, the ER-CALUX assay is less cumbersome and time consuming, as it is not necessary to transfect DNA for each experiment. Problems with differences in quality of DNA and changes in luciferase activity due to cell culture conditions found with transient transfections (Zacharewski, 1997) are therefore avoided. A number of yeast-based assays have been developed to assess estrogenic substances which are very sensitive and responsive (Arnold *et al.*, 1996; Routledge and Sumpter, 1997). Unlike the ER-CALUX assay, however, lack of antagonism of ER-mediated reporter gene expression by anti-estrogens has been demonstrated

in yeast-based assays (Lyttle *et al.*, 1992; Kohno *et al.*, 1994). Permeability of compounds through yeast cell wall may also be a problem not encountered in the ER-CALUX assay (Legler, in prep.). In comparison to cell proliferation assays with estrogen responsive cell lines like MCF-7, such as the E-SCREEN (Soto *et al.*, 1995), the ER-CALUX assay is more specific, rapid, sensitive and responsive.

Table 3: Comparison of detection limit (d.l.), EC50 and responsiveness to estradiol (fold induction) in recombinant receptor/reporter gene assays with stable or transient transfection of estrogen-receptor mediated reporter genes in mammalian cell lines

Reference	Transfection	DNA construct	Cell line	d.l. (pM)	EC50 (pM)	Induc-tion
this study	stable	pEREtata-Luc	T47-D	0.5	6	100
Pons et al, 1990	stable	pVit-tk-Luc	MCF-7	n.s.	20	5
Kramer et al., 1997	stable	pVit-tk-Luc	MCF-7	n.s.	1.5	2.2
Balaguer et al., 1996	stable	pGal4HEGO/ p17m5-G-Luc	HeLa	18	40	10
Zacharewski et al., 1995	transient	pGal4HEGO/ p17m5-G-Luc	MCF-7	n.s.	20	50
White et al., 1994	transient	pEREBLCAT	MCF-7	n.s.	n.s.	6
Jobling et al., 1995	transient	pERE-tk-Luc	MCF-7	n.s.	50	10

n.s. = data not shown

We believe that the ER-CALUX assay can form a valuable contribution to a careful selection of *in vitro* and *in vivo* assays used to identify and determine the (anti-)estrogenicity of compounds. The ER-CALUX assay is also currently being used to identify (anti-)estrogenic substances in complex environmental mixtures, such as sediment and water extracts (Legler, in prep.). Of course, the *in vitro* ER-CALUX assay cannot completely reflect complex *in vivo* events, such as the pharmacokinetics, metabolism and accumulation of a compound. T47D.Luc cells possess some metabolic capabilities, such as the cytochrome P450 enyzmes involved in the hydroxylation of estrogens and xenobiotics. For example, P450IA (data not shown, Kuil *et al.*, 1998, Spink *et al.*, 1998); P4501B (Spink *et al.*, 1998) and 17β-

hydroxysteroid dehydrogenase (Piao et al., 1997) activity has been reported in T47D cells. In an *in vitro* assay, cross-talk with other mechanisms not directly involving interaction with the ER signal transduction pathway may be also missed. In addition, caution should be exercised when using a breast cancer cell line expressing both ERα and ERβ subtypes for the prediction of tissue-specific effects. Tissues such as prostrate and ovary have been found to contain prominent ERβ expression (Kuiper *et al.*, 1997). Differences in the binding affinities of compounds, such as phytoestrogens, as well as in the transcriptional activity for both ER subtypes have been demonstrated (Kuiper *et al.*, 1998). Further research can determine whether estrogenic potency predicted in the ER-CALUX assay using human T47D.Luc cells may be extrapolated to other species, such as fish, birds and reptiles. If not, recombinant reporter gene assays should be developed using species specific estrogen receptors in estrogen-sensitive cell lines from various species. To address many of these questions, biological validation is currently underway in fish to compare the reproductive effects of (mixtures of) estrogens *in vivo* to effects measured by the ER-CALUX assay.

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Chapter 3

Detection of estrogenic activity in sediment-associated compounds using *in vitro* reporter gene assays

submitted to *Toxicological Sciences*

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Abstract

Sediments may be the ultimate sink for (xeno-)estrogenic compounds in the aquatic environment. *In vitro* reporter gene assays can provide useful and rapid means to assess the total estrogenic activity in complex mixtures such as sediment extracts. To determine a suitable assay to test environmental mixtures for estrogenic potency, a recently developed estrogen receptor-mediated luciferase reporter gene (ER-CALUX) assay in stably transfected T47D human breast cancer cells was compared with a recombinant yeast screen. Following exposure to the (xeno-)estrogens 17β-estradiol (E2), nonylphenol and o,p'DDT, the ER-CALUX assay was more sensitive than the recombinant yeast screen, with an EC50 of 6 pM E2 compared to 100 pM in the yeast screen. In addition, recombinant yeast cells were unable to distinguish substances with an anti-estrogenic mode of action, as the anti-estrogens ICI 182,780, hydroxytamoxifen and tamoxifen were agonistic in the yeast. Complex mixtures of compounds present in sediments from various marine locations in The Netherlands were tested for estrogenic activity in the ER-CALUX assay. Acetone-soluble polar extracts of sediments showed higher estrogenic potency than hexane-soluble non-polar extracts. Polar extracts from sediments obtained from industrialized areas such as the Port of Rotterdam showed the highest estrogenic potency of the sediments tested (up to 40 pmol estradiol equivalents per gram sediment). The estrogenic activity of individual chemicals that may be found in sediments, including alkylphenols ethoxylates and carboxylates, phthalates, and pesticides, was tested. Increasing sidechain length of various nonylphenol ethoxylates resulted in decreased estrogenic activity. Of the phthalates tested, butylbenzylphthalate was the most estrogenic, though with a potency approximately 100,000 times less than E2. Though o,p'DDT and o,p'DDE were estrogenic in the ER-CALUX assay, the organochlorine herbicides atrazine and simazine were not estrogenic. As metabolic activation may be required to induce estrogenic activity in some sediment-associated compounds, a metabolic transformation step was added to the ER-CALUX assay. Parent compounds were incubated with liver microsomes obtained from PCB-treated rats and metabolites were extracted and tested in the ER-CALUX assay. Results indicated that metabolites of E2, NP and Bisphenol A were less active, while metabolites of methoxychlor were more estrogenic than the parent compound following microsomal incubations.

Introduction

In the aquatic environment, exposure of organisms to substances with an estrogenic mode of action has been linked to endocrine effects observed in wild populations of male fish, such as vitellogenin induction and feminized reproductive organs (reviewed by Tyler *et al.*, 1998). Chemical analysis has revealed the presence of various substances that possess estrogenic activity in wastewater treatment plant (WTP) discharges, which may form a main source of estrogenic substances in the aquatic environment. These include natural estrogens, such as 17β-estradiol (E2), synthetic estrogens such as the contraceptive pill component, 17α-ethynylestradiol (EE2), as well as estrogenic chemicals of anthropogenic origin ("xeno-estrogens"), such as alkylphenols (Shore *et al.*, 1993, Desbrow *et al.*, 1998, Belfroid *et al.*,

1999a, Snyder *et al.*, 1999). The use of chemical analysis is essential to determine the exposure concentrations of (xeno-)estrogens to organisms and help to assess the potential risk of (xeno-)estrogens in the environment. This type of analysis, however, is limited in that only known substances are analysed, mixture interaction is not taken into account, and the biological effects of the (xeno-)estrogen or mixtures can not be predicted.

The use of *in vitro* bioassays based on the mechanism of action of estrogens can be very useful to rapidly and inexpensively determine the total biological potency of individual substances as well as mixtures of chemicals. Two examples of *in vitro* reporter gene bioassays for determining estrogenic effects include the recently developed estrogen receptor (ER)-mediated Chemical Activated Luciferase gene eXpression (ER-CALUX) assay (Legler *et al.*, 1999) and the recombinant yeast screen (Routledge and Sumpter, 1996). The ER-CALUX assay uses T47-D human breast adenocarcinoma cells expressing endogenous ER α and β, which are stably transfected with an estrogen-responsive luciferase reporter gene. Exposure of stably transfected T47-D cells to (xeno-)estrogens results in transactivation of the ER and consequent induction of the luciferase gene, which is easily assayed by lysing cells and adding the substrate luciferin, and measuring light output. In the recombinant yeast screen, yeast cells have been stably transformed with human ER-α cDNA and an ERE-regulated expression plasmid (*lac-Z*). Interaction of an estrogenic compound with the ER results in expression of the reporter gene *lac-Z* and secretion of the enzyme β-galactosidase in the yeast medium. Both the ER-CALUX assay and recombinant yeast screen are rapid and easy to perform in a microtiter, high-throughput manner.

Mixtures of (xeno-)estrogens released in the environment via domestic and industrial effluents, as well as from agricultural runoff, may accumulate in sediments. Sediments may be the ultimate sink for many persistent chemicals released in aquatic systems, and may form a source of exposure to aquatic organisms. This may be particularly important for the Netherlands, which forms the sedimentation basin for major European rivers such as the Rhine, Meuse and Scheldt. Therefore, the aim of this study was to determine the *in vitro* estrogenic activity in sediments sampled from various locations along the Dutch coast. In order to choose the most suitable *in vitro* assay for this purpose, the ER-CALUX assay was first compared with the recombinant yeast screen for responses to estrogens and anti-estrogens. In addition, a number of alkylphenol ethoxylates and phthalates detected in Dutch sediments (Belfroid *et al*, 2000), as well as persistent organochlorine pesticides which may be found in sediments (DDT derivatives and chloro-s-triazine-derived herbicides) were tested for estrogenic potency in the ER-CALUX assay. Because sediment-associated compounds may also only be estrogenic following metabolic activation, an additional goal of this study was to include a metabolic transformation step in the ER-CALUX assay in order to determine the estrogenic potential of metabolites as well as parent compounds. The model (xeno-)estrogens estradiol, bisphenol A, nonylphenol and methoxychlor were pre-incubated with liver microsomes from rats treated with the PCB mixture Aroclor 1254, which induces a broad spectrum of liver enzymes (especially CYP 1A), and tested in the ER-CALUX assay. Our results indicate that a simple metabolism step can be added to the ER-CALUX assay, allowing for the measurement of the impact of metabolic transformation on the estrogenic activity of (xeno-)estrogens.

Materials and Methods

Chemicals

17β-estradiol (E2, 99%), methoxychlor (95%), tamoxifen (99%), and ethanol (100%, p.a.) were purchased from Sigma Chemicals. 4-nonylphenol (92.7%) was purchased from Fluka. Bisphenol A (99%) was from Aldrich. The nonylphenol ethoxylates (NpnEO), nonylphenol carboxylates (NpnEC) and octylphenol ethoxylates (OP 8/9EO) were kindly provided by Dr. P. de Voogt, University of Amsterdam, The Netherlands. ICI 182,780 and 4-hydroxy-tamoxifen (OHT) were a kind gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, U.K. The organochlorine pesticide o,p'-dichlorodiphenyl trichloroethane (o,p'DDT) was kindly provided by the Dutch State Institute for Quality Control of Agricultural Products (RIKILT-DLO). Simazine, atrazine, desethylatrazine, deisopropylatrazine and o,p'DDE were a kind gift from E. van der Velde, National Institute of Public Health and the Environment, Bilthoven, The Netherlands. Dimethylphthalate (DMP, 99.5%), di-ethylhyexyl phthalate (DEHP; 98%), 4-octylphenol (OP, 99%) and dimethyl sulfoxide (DMSO, 99.9%, spectrophotometric grade) were purchased from Acros. Diethyl phthalate (DEP, 99%) was from Alfa. Dibutyl phthalate (DBP; 99%) and butylbenzyl phthalate (BBP, 98%) were purchased from TCI. Solvents used for extractions (hexane, acetone, dichlormethane, methanol and diisopropyl ether; Merck) were of p.a. or HPLC quality.

ER-CALUX assay procedure

Culture of the T47D cell line which is stably transfected with the ER-regulated pEREtata-Luc DNA construct (T47D.Luc cells) is described elsewhere (Legler *et al.*, 1999). For the ER-CALUX assay, T47D.Luc cells were plated in clear plastic 96 well plates (Nucleon, Denmark) at a density of 5000 cells in 0.1 ml Dulbeccos's modified Eagle's medium and Ham's F12 (DF) medium without phenol red with 5% dextran-coated charcoal treated fetal calf serum FCS (DCC-FCS) per well. Following 24 hours incubation, medium was renewed and the cells incubated for another 24 hours. The medium was then removed and the cells were dosed in triplicate by addition of the dosing medium containing the chemical or extract to be tested dissolved in ethanol or DMSO (max. 0.2%). Control wells, solvent control wells and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. Following 24 hours treatment, medium was removed and cells were lysed in 50 µl triton-lysis buffer, pH. 7.8 (containing 1% triton X-100, 25 mM glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT) for a minimum of 1 hour with gentle shaking at 4°C. A sample of 25 µl lysate was then transferred to a black 96 well plate (Costar) and 25 µl of luciferin solution (Luclite, Packard) was added per well. Luciferase activity was assayed in a scintillation counter (Top Count, Hewlett-Packard) for 0.1 minute per well.

Recombinant yeast screen

Yeast cells stably expressing the human ER and ERE-lacZ expression plasmid were kindly provided by Glaxo Group Research Ltd, United Kingdom. Yeast culture and galactosidase measurement was carried out mainly according to the assay procedure of Routledge and Sumpter, 1996. Growth and assay media were prepared as described previously (Routledge

and Sumpter, 1996). Briefly, 0.25 ml of a concentrated recombinant yeast stock was added to 45 ml growth medium and incubated overnight at 28°C in a shaking incubator (250 rpm) until an optical density (OD) of 1.0 (640 nm) was reached. Assay medium was prepared by adding 0.5 ml of the substrate chlorophenol red-B-D-galactospyranoside (CPRG, Boeringer Mannheim) to 50 ml fresh growth medium, and adding 2 ml yeast from the overnight culture. Clear plastic 96 well plates (Costar) were seeded with 200 µl per well of the assay medium containing yeast, using a 8-well multichannel pipettor, in a laminar air flow cabinet. Stock solutions and dilutions of test compounds were prepared in DMSO and added directly to the wells containing the yeast, to a maximum concentration of 2% DMSO (i.e. 4 µl in 200 µl). Each dilution was tested in triplicate. Each assay included a standard curve for E2 (1 to 10000 pM) and each plate contained triplicate solvent control and E2 calibration points (100 and 1000 pM). For experiments involving co-treatment of compounds (e.g. E2 with anti-estrogens), test compounds were added together so that the final solvent concentration did not exceed 2%. Plates were then covered with a lid, and shaken vigorously on a plate shaker for 5 minutes. Plates were incubated for 3 days at 32°C, with daily 5 minute shaking of plates. Substrate conversion (color development) was measured at 540 nm and cell growth at 640 nm in a 96 well plate spectrophotometer (Spectromax). As neglible absorbance was measured in blank wells containing medium alone, the formula used to calculate absorbance per well was OD540 – OD640.

Sediment extraction

A preliminary study was carried out to determine if estrogenic activity was found in non-polar or polar extracts of sediments. A sediment sample from a relatively polluted area located in the Dutch Wadden Sea near a freshwater outlet was dried overnight at 30°C. A dried sediment sample of 14.05 g was extracted with hexane:acetone (1:1) for 2 hours in a Soxtec apparatus (miniaturized Soxhlet, Tecator, The Netherlands). The hexane/acetone fraction was then evaporated to approximately 2 ml. After sulphur removal using tetrabutyl ammonium sulfite (TBA) (De Voogt *et al.*, 1990), the extract was transferred to hexane resulting in a precipitate of acetone soluble components. The hexane fraction was then split in two equal portions. One portion was filtered over a 1 g Na_2SO_4 column, evaporated and taken up in 50 µl DMSO, resulting in a non-polar fraction. The other portion was subjected to further clean-up over a multilayer acid-base silica column to remove labile components (Murk *et al.*, 1996), evaporated and taken up in 100 µl DMSO. This extraction procedure resulted in a stable non-polar fraction. The precipitate of acetone soluble components mentioned above was redissolved in acetone. This acetone fraction was filtered over a 1 g Na_2SO_4 column, evaporated and taken up in 100 µl ethanol, resulting in a polar fraction.

To determine estrogenic potency in polar fractions of a number of field samples, sediment was sampled from 12 marine locations in the Netherlands (De Boer *et al.*, 2000). Dried sediment samples of 5 g were extracted with hexane:acetone (1:1) for 2 hours in a Soxtec apparatus (Tecator, The Netherlands). After sulphur removal as described above, the extract was transferred to hexane resulting in a precipitate of acetone soluble components. This precipitate (representing the polar fraction) was redissolved in acetone, filtered over a 1 g Na2SO4 column, evaporated and taken up in 50 µl DMSO. Stocks were diluted 100 times

in DMSO and tested in the ER-CALUX assay according to the procedure described above. These extract dilutions demonstrated no toxicity, determined by microscopic observations of the cells.

Microsomal biotransformation studies

For *in vitro* microsomal biotransformation studies, parent compounds were incubated with rat liver microsomes according to the method of Morse *et al.*, 1995. Microsomes were collected as described in Morse *et al*, 1993, from rats treated with two daily interperitoneal injections of 100 mg/kg Aroclor 1254. Microsomal incubation mixtures were prepared in glass tubes on ice and contained 10 µl of a 10 mM stock concentration of E2, methoxychlor, nonylphenol or bisphenol A (final concentration 100 µM) in a total volume of 900 µl of 0.1 M Tris-HCl buffer, pH 7.5. Microsomal protein concentration in the incubation mixture was 1 mg/ml. The incubation mixtures were vortexed and pre-incubated for 2 minutes at 37°C. The reaction was initiated by the addition of 100 µl NADPH (Boeringer Mannheim) in Tris-HCL buffer to a final concentration of 1 mM NADPH. In parent compound controls, only 100 µl of Tric-HCl buffer was added. Following 1-hour incubation at 37°C in a shaking water bath, reactions were stopped by the addition of 2 ml ice-cold methanol and vortexing for 10 sec. The tubes were then centrifuged 5 min. at 3000 rpm and the supernatant was transferred to a clean tube. Parent compounds and metabolites were extracted three times with 2 ml diisopropyl ether by vortexing 1 minute, centrifuging 3 min. at 2000 rpm and transferal of the diisopropyl fraction. The ether extracts were pooled and dried at room temperature under a gentle N2 stream, transferred to glass conical vials, and taken up in 25 µl ethanol. Extracts were diluted and tested in the ER-CALUX assay as described above.

Data analysis

To determine the EC10 and EC50 of E2 in the ER-CALUX and recombinant yeast assay, a complete standard curve was included in each assay. The standard curve was fitted using a cumulative fit function using SlideWrite 4.0 for Windows. The EC50 for E2 and xeno-estrogens was calculated by determining the concentration at which 50% of the maximum reporter gene activity was reached using the cumulative fit equation. In some cases, an EC50 value could not be calculated, as a plateau in reporter gene activity was not reached. In this case, an EC10 was calculated by determining the reporter gene induction by E2 at 10% the maximum response, and the concentration of the (xeno-)estrogen at this induction level was interpolated in the curve. The detection limit was calculated as the reporter gene activity elicited by the solvent control plus three times the standard deviation.

Estradiol equivalency factors (EEFs) were calculated by dividing the EC50 or EC10 of E2 by that of the xeno-estrogen. In order to determine estradiol equivalents (EEQs) in mixtures, the reporter gene response by sediment extracts was interpolated in the lower linear range (between 1 and 6 pM) of the corresponding E2 standard curve for the same assay. Data shown are representative of a minimum of three independent assays. Compounds were tested in triplicate; error bars represent standard deviation of the mean.

Results

Comparison ER-CALUX and recombinant yeast screen

Comparison of the E2 standard lines in the ER-CALUX and recombinant yeast screen demonstrated that the ER-CALUX is about a factor 20 more sensitive (Figure 1). The calculated detection limit and EC50 for the ER-CALUX was 0.5 and 6 pM, respectively, and for the yeast screen 10 pM and 100 pM, resp. The maximum fold induction in the yeast screen (at 1000 nM) was 8-16 fold relative to solvent controls. In the ER-CALUX, maximum fold induction was found at 30 pM and was 75 to 100 fold (Figure 1). The reporter gene induction by two known xeno-estrogens, 4-nonylphenol (NP) and o,p'DDT, was tested in the ER-CALUX and yeast screen assays. The EC50 in the ER-CALUX for NP and o,p'DDT is 260 and 660 nM, respectively (Legler *et al.*, 1999). In the yeast recombinant screen, however, EC50s for these compounds were about 6 to 10 times higher, with NP demonstrating an EC50 of 2000 nM and o,p'DDT 3600 nM (data not shown).

Reporter gene induction by the anti-estrogens ICI 182,780, tamoxifen and 4-hydroxytamoxifen (OHT) was tested with and without co-administration of E2 at the EC50 level (Table 1; Figure 1). In the ER-CALUX assay, OHT and ICI 182,780 alone showed no reporter gene induction (Table 3). Tamoxifen was weakly estrogenic at high concentrations in the ER-CALUX assay, as a concentration of 1000 nM resulted in luciferase induction about 11% that of 6 pM E2 (Table 1). In the recombinant yeast screen, the three anti-estrogens induced reporter gene activity when tested alone without co-treatment with E2. At concentrations of 1000 nM, OHT and tamoxifen induced β-galactosidase activity over 300% that of activity elicited by 100 pM E2. When tested at 10000 nM, the estrogenic activity of OHT decreased, though it still induced reporter gene induction to about 60% that of 100 pM E2, or about 5 fold above solvent controls (Figure 1). ICI 182,780 demonstrated dose-response related agonistic activity in the yeast screen, with 10000 nM resulting in reporter gene activity of 770% that of 100 pM E2. In experiments in which cells were co-treated with 6 pM E2 in the ER-CALUX assay, OHT and ICI 182,780 at 10 nM already completely abolished the luciferase induction by E2 (Table 1). Tamoxifen demonstrated anti-estrogenic working at concentrations at or above 100 nM in the ER-CALUX assay. In the recombinant yeast screens, none of the anti-estrogens tested could antagonize the reporter gene induction by 100 pM E2. With the exception of OHT at the highest concentrations (10,000 nM), treatment of E2 with anti-estrogens suggested that these compounds worked as agonists rather than antagonists in the recombinant yeast screen (Table 1).

Because of the differences in sensitivity to (xeno-)estrogens and anti-estrogens between the ER-CALUX and recombinant yeast assays, further studies were carried out with the ER-CALUX assay.

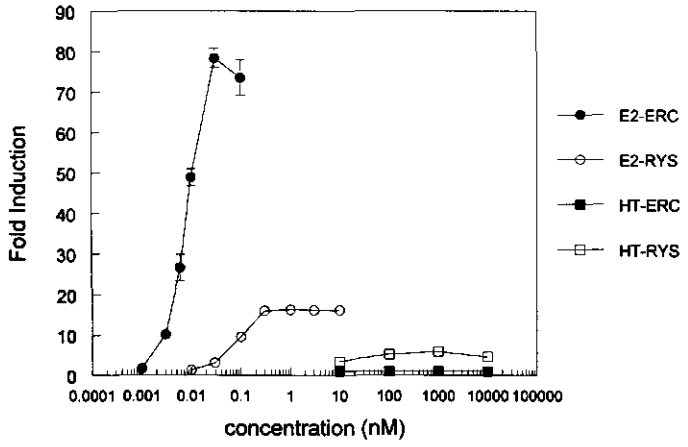

Figure 1: Effects of estrogen and anti-estrogens in the ER-CALUX assay (ERC) and recombinant yeast screen (RYS). T47D.Luc cells were exposed for 24 hours and yeast cells for 72 hours to estradiol (E2) and 4-hydroxytamoxifen (HT). Response is given as reporter gene induction relative to solvent controls (fold induction).

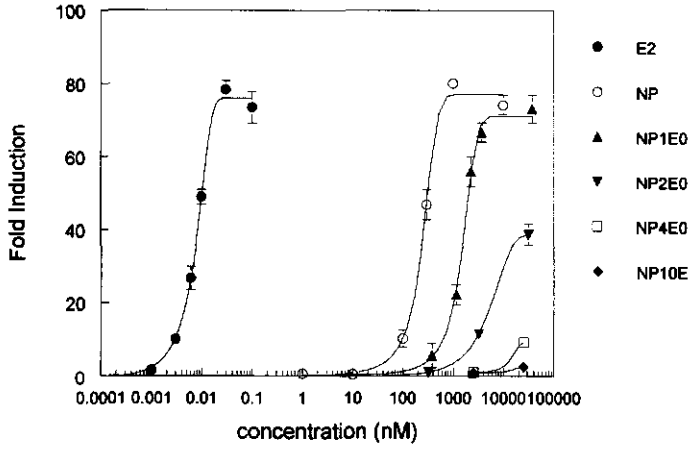

Figure 2: Estrogenic activity (fold induction relative to control) of estradiol (E2), nonylphenol (NP) and nonylphenol ethoxylates (NPnEO) in the ER-CALUX assay using T47D.Luc cells (24 hour exposure).

Table 1: Response of anti-estrogens in the ER-CALUX and recombinant yeast screen assays. Anti-estrogens were tested alone or with co-treatment with estradiol at the EC50 level of E2 (6 pM for ER-CALUX, 100 pM for yeast). Reporter gene induction is expressed at average percentage relative to E2 (standard deviation bracketed)

			Co-treatment with Estradiol	
	Reporter Gene Induction (%)		Reporter Gene Induction (%)	
Conc. (nM)	ER-CALUX	Yeast screen	ER-CALUX	Yeast screen
4-hydroxy-tamoxifen				
10	0.5 (0)	72 (2)	0.0	317 (40)
100	0.4 (0)	290 (83)	0.0	353 (26)
1000	0.4 (0)	339(10)	0.0	347 (13)
10000	0.0 (0)	59 (19)	0.0	102 (21)
ICI 182,780				
10	0.0	4 (0.2)	0.0	103 (11)
100	0.0	13 (1.0)	0.0	202 (2)
1000	0.7 (0)	33 (6.1)	0.0	319 (18)
10000	0.0 (0)	773 (11.2)	0.0	774 (8)
Tamoxifen				
10	0.2 (0.0)	7 (0.2)	52 (2)	193 (25)
100	7 (0.3)	13 (1.4)	5 (0.6)	261 (14)
1000	11 (0.1)	397 (20.1)	8 (0.4)	606 (5)
10000	*	*	*	*

*cell lysis observed

Estrogenic potency of mixtures of estrogenic compounds in sediments

Sediment sampled from a contaminated sight in the Dutch Wadden Sea was chosen as a model sediment to determine suitable extraction procedure for sediment samples as. Polar acetone-soluble extracts from this sediment demonstrated higher estrogenic potency in the ER-CALUX assay than non-polar hexane extracts. The polar extract contained 5 pmol EEQ/g sediment, whereas the both the total non-polar and stable non-polar extracts from this sediment contained considerably less estrogen equivalents (0.3 and 0.4 pmol EEQ/g sediment, respectively). Further determination of estrogenic potency in sediments sampled from 12 locations along the Dutch coast was therefore carried out in polar extracts. Sediment samples taken from the port of Rotterdam and one location along the Dutch coast (IJmuiden Harbour) showed the highest estrogenic activity ranging from 22-38 pmol/g sediment in comparison to locations in the port of Amsterdam and two relatively unpolluted 'reference' areas (5-7 pmol EEQ/g sediment) (Table 2).

Table 2: Estradiol equivalents (EEQ) following 24 hour treatment of T47D.Luc cells with polar acetone-soluble extracts of sediments collected from marine locations in the Netherlands

Location	EEQ[1]	Location	EEQ
Port of Rotterdam:		**Port of Amsterdam:**	
Nieuwewaterweg Benelux Tunnel	38.4 (9.5)	IJmuiden Harbour (inner)	7.3 (0.5)
Nieuwewaterweg Splitsingdam	22.0 (4.5)	North Sea Canal km 10	5.4 (1.5)
Dutch Coast:		North Sea Canal km 18	7.1 (2.9)
Loswal North km 53	6.4 (1.5)	North Sea Canal km 29	6.0 (1.2)
Loswal North km 12	4.5 (0.6)	**"Reference" areas:**	
Noordwijk km 2	15.0 (5.7)	Lake IJssel	7.5 (0.6)
Ijmuiden Harbour (outer)	27.1 (10.1)	Eastern Scheldt	4.9 (0.8)

[1]pmol/g sediment, average ± std

Estrogenic potency of xeno-estrogenic substances in ER-CALUX

The ER-CALUX was further characterized in this study for response by xeno-estrogens that have been detected in sediments in the Netherlands such as alkylphenols and phthalates (Belfroid *et al.*, 2000). In addition, a number of persistent organochlorine pesticides that may be found in sediments were tested. When testing nonylphenol, octylphenol, nonylphenol- and octylphenol ethoxylates in the ER-CALUX, increasing size of the ethoxylate sidechain resulted in a decrease in estrogenic activity, with luciferase induction decreasing from 80-fold for NP1EO to 9-fold for NP4EO and no induction by NP10E0 (Figure 2). Estrogenic potency of the nonylphenol exothylates was about 10^{-6} to 10^{-7} that of E2 (Table 3). The final alkylphenol degradation products, NP and OP, demonstrated highest estrogenic potency, with an EEF between 10^{-5} to 10^{-6} resp. relative to E2 (Table 3). The nonylphenol carboxylates (NP1EC and NP2EC) tested, as well as the octylphenol ethoxylates (OP 8/9EO) failed to induce luciferase activity (Table 3). Compounds tested at concentrations exceeding 10 μM were cytotoxic. Of the phthalates tested, only butylbenzylphthalate (BBP) showed a complete dose-response curve, though at concentrations 100,000 higher than E2 (Figure 3, Table 3). Diethyphthalate (DEP) and dibutylphthalate (DBP) were weakly estrogenic, demonstrating slight luciferase activity (about 10-fold induction) at concentrations of 10 μM (Figure 3). Dimethylphthalate (DMP) and diethylhexylphtalate (DEHP) showed no estrogenic actitivity in the ER-CALUX assay. Of the pesticides tested, only o,p'DDE and o,p'DDT showed estrogenic activity whereas the chloro-s-triazine-derived herbicides simazine, atrazine, desethylatrazine, and deisopropylatrazine failed to induce luciferase activity (Table 3).

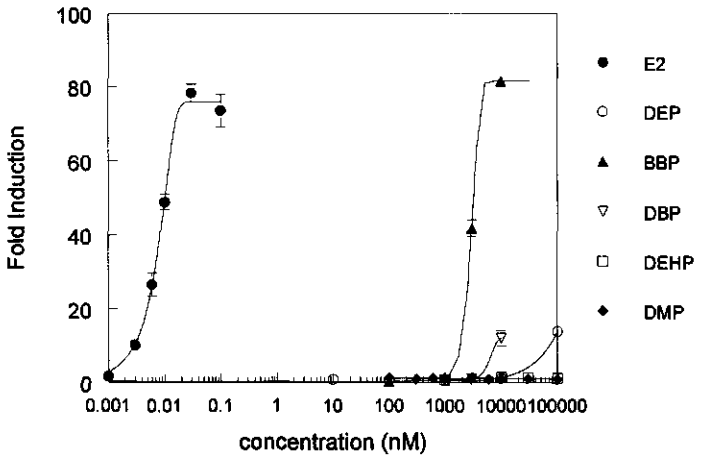

Figure 3: Estrogenic activity (fold induction relative to control) in T47D.Luc cells exposed for 24 hours to estradiol (E2), and phthalates: diethylphthalate (DEP), butylbenzylphthalate (BBP), dibutylphthalate (DBP), dimethylphthalate (DMP) and diethylhexylphthalate (DEHP).

Microsomal biotransformation in ER-CALUX assay

In an attempt to determine the estrogenic potential of metabolites as well as parent compounds, a microsomal biotransformation step was included in the ER-CALUX assay. The biotransformation step involved *in vitro* metabolism of parent compounds using liver microsomes from rats pre-treated with the PCB mixture Aroclor 1254, which induces a broad range of metabolic enzymes in rat liver, including hepatic P450 1A and 2B enzymes (Safe, 1984). Following 24-hour exposure, metabolites of estradiol, (Figure 4a), nonylphenol and bisphenol A (data not shown) obtained by microsomal incubations showed less estrogenic potency than parent compounds. Metabolites of methoxychlor, however, microsomes showed about two to three times higher estrogenic potency than the parent compound (Figure 4b).

Table 3: Estradiol equivalence factors (EEF) calculated for xeno-estrogens in the ER-CALUX assay. EEFS are ratio $EC50_{17\beta\text{-estradiol}}$:$EC50_{compound}$, unless indicated otherwise.

Compound	EEF	Compound	EEF
17β-estradiol	1		
Alkylphenols:		**Phthalates:**	
4-nonylphenol	$2.3*10^{-5}$	Dimethylphthalate	n.c.
4-octylphenol	$1.4*10^{-6}$	Diethylphthalate	$3.2*10^{-8**}$
NP1E0	$3.8*10^{-6}$	Dibutylphthalate	$1.8*10^{-8**}$
NP2E0	$1.1*10^{-6}$	Butylbenzylphthalate	$1.4*10^{-6}$
NP4E0	$1.1*10^{-7**}$	di(2-ethylhexyl)phthalate	n.c.
NP10E0	n.c[#]	Dioctylphthalate	n.c
NP1EC	n.c.		
NP2EC	n.c.		
OP8/9EO	n.c.		
Pesticides:			
Op'DDT	$9.1*10^{-6}$		
O,pDDE	$2.3*10^{-6}$		
Simazine	n.c.		
Atrazine	n.c.		
Desethylatrazine	n.c.		
Deisopropylatrazine	n.c.		

[#]not calculated; no luciferase induction to the EC10 level observed
[**]calculated based on EC10

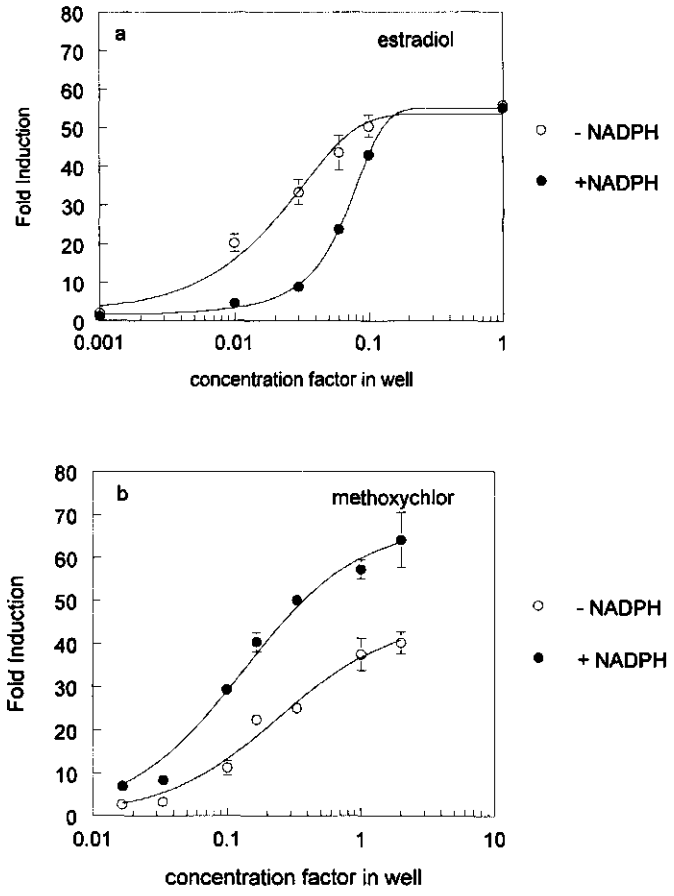

Figure 4: Luciferase activity in T47D.Luc cells exposed for 24 hours to dilutions of (A) estradiol and (B) methoxychlor without (open circles; -NADPH) or with (closed circles; +NADPH) in vitro biotransformation. Compounds were pre-incubated with liver microsomes from Aroclor 1254 treated rats and extracted.

Discussion

Comparison ER-CALUX assay and recombinant yeast screen

Both the ER-CALUX and recombinant yeast screen assays provide a simple and rapid means to screen substances for their ER transcriptional activity *in vitro*. The recombinant yeast screen has a number of advantages, including the absence of other endogenous receptors and consequent lack of complex interaction between the ER and other receptors (Routlegde and Sumpter, 1996). The yeast cells grow in a medium devoid of hormones, thereby ensuring low background levels. The ease of use of the recombinant yeast screen is another definite advantage, as the product of the reporter gene is secreted in the medium and no cell lysis is necessary. In comparing the recombinant yeast screen with the ER-CALUX assay, however, differences in responses to (xeno-)estrogens and anti-estrogens are evident. Firstly, a difference in the sensitivity was found between the ER-CALUX and recombinant yeast screen, demonstrating that the ER-CALUX can detect estrogens at lower concentrations. For E2, nonylphenol and o,p'DDT, a difference of about 6 to 20 fold was found between the EC50 in the ER-CALUX and the yeast screen. Other yeast-based estrogen screens have also reported different ligand potencies when compared to mammalian *in vitro* assays (Zacharewski, 1997). There may be a number of explanations for this difference in sensitivity between the two assays. One explanation is that permeability may considerably differ between the yeast and mammalian cell membranes, with the yeast cell wall allowing fewer substances to passively diffuse or exhibiting selective uptake of some compounds. Lyttle and coworkers (1992) developed a hyperpermeable strain of yeast by selecting the yeast on increasing concentrations of the antibiotic nystatin, which forms a trans-membrane canal with cholesterol, thereby producing a "leaky" membrane through which more ligands can pass. Using this hyperpermeable strain of yeast, they showed that less hydrophilic estrogen conjugate E2-valerate was more "estrogenic" than in wild type strains transformed with hER and hER-regulated reporter genes (Lyttle *et al.*, 1992). Other mechanisms that may be involved in the difference in sensitivity between the yeast- and mammalian-based assays include differences in cellular transcription factors (Tora *et al.*, 1989; Halachmi *et al.*, 1994), multiple drug resistance (Dexter *et al*, 1994) and endogenous yeast estrogen binding proteins (Feldman *et al*, 1982). Another possibility is a difference in the solubility and availability of compounds in the assays. In the ER-CALUX assay, cells are exposed in medium containing 5% serum. Proteins in serum may act as carriers for ligands, thereby increasing their bioavailability. No such carrier molecules are present in the water-based recombinant yeast medium. Arnold and colleagues (1996) demonstrated that addition of albumin, sex hormone binding globulins, or serum to yeast medium induced greater reporter gene activity by the synthetic estrogen diethylstilbestrol and xeno-estrogens o,p'DDT and octylphenol.

The difference in response to anti-estrogens in the ER-CALUX assay and recombinant yeast screen forms another important distinction between the two assays. While classical anti-estrogens such as ICI 182,780 and tamoxifen are potent inhibitors of E2-mediated reporter gene induction in the ER-CALUX assay, these compounds showed agonistic, rather than antagonistic, effects in the recombinant yeast screen (Table 3). Other studies have also reported the agonistic effects of ICI 182,780 and/or tamoxifen in yeast

(Lyttle *et al.*, 1992; Shiau *et al.*, 1996; Beresford *et al.*, 2000; Dudley *et al.*, 2000). In another study with the recombinant yeast screen, hydroxytamoxifen has also been shown to be a weak estrogen agonist (Beresford *et al.*, 2000). However, in contrast to the results present in our study, OHT was able to antagonize E2-mediated β-galactosidase activity, either partially (Beresford *et al.*, 2000) or completely in dose-response related manner (Sohoni and Sumpter, 1998). The reason why OHT did not demonstrate antagonistic effects in our study is unclear and may be due to differences in yeast cell culture or administration of test compounds. However, the fact that contradicting results have been reported for OHT in the recombinant yeast screen suggests that OHT is not a clear anti-estrogen in the yeast screen. A number of hypotheses have been offered to explain the lack of anti-estrogenic effects in yeast, including reduced permeability of the yeast cell wall to these compounds (Zysk *et al.*, 1995) or lack of appropriate repressor compounds in yeast necessary for antagonism (Gaido *et al.*, 1997). Data presented in a recent study favor the hypothesis that anti-estrogens work in mammalian systems by inducing ER down-regulation (Dudley *et al.*, 2000), which is not attained in the yeast system. The difference in ability to differentiate between estrogen agonists and antagonists between the ER-CALUX and yeast recombinant screen should be kept in mind, particularly when testing complex mixtures for total estrogenic potency. Importantly, because the yeast screen is less sensitive to some (xeno-)estrogens than the ER-CALUX assay, larger samples would have to be processed when testing environmental matrices. Accordingly, when testing estrogenic potency in water and effluent, it was shown that considerably more material was needed in the yeast screen to elicit an estrogenic response than in the ER-CALUX assay (Murk *et al.*, 2000).

In vitro estrogenic potency of sediment extracts

The ER-CALUX assay can be successfully applied to provide an indication of the estrogenic potency in complex mixtures, such as extracts from environmental matrices. Polar (acetone-soluble) extracts of sediments contained higher estrogenic activity than non-polar (hexane-soluble) extracts, demonstrating that biologically active estrogenic compounds may be of a more hydrophilic nature. Polar extracts of sediments sampled from Rotterdam Harbour showed the highest estrogenic activity of the twelve locations tested (Table 1). Though detailed chemical analysis of the levels of (xeno-)estrogens has not been carried out on these extracts, levels of some chemicals with estrogenic potency, such as polybrominated diphenylethers and polycyclic aromatic hydrocarbons (PAHs) have been measured in these sediments (De Boer *et al.*, 2000). The highest concentrations of PAHs and PBDEs at the twelve sediment locations were found in Rotterdam Harbour sediments (De Boer *et al.*, 2000). Some chemicals in these groups have previously been shown to be estrogenic in the ER-CALUX assay, such as the PAH benzo[a]pyrene (Legler *et al.*, 2000c) and some PBDEs (Meerts *et al.*, 2000). Other evidence of the presence and effects of (xeno-)estrogenic compounds in Rotterdam Harbour sediments was found in mesocosm experiments in which female flounder exposed for three years in tanks containing harbour sediment showed premature vitellogenesis (Janssen *et al*, 1997). These results demonstrate that (xeno-)estrogenic substances are not only of importance in the water phase, but may also accumulate in sediments in the aquatic environment. Therefore, more attention should be given to the

estrogenic effects of compounds that may be available to aquatic organisms via food and through contact with particulate matter and sediment.

Estrogenic activity of environmentally relevant xeno-estrogens

Recent studies have shown that akylphenol ethoxylates and phthalates are commonly detected in sediments in The Netherlands (Belfroid *et al.*, 2000). Some of these substances are also estrogenic in the ER-CALUX assay. The degradation products of the alkylphenols with no (nonylphenol) or short sidechain ethoxylates (NP1E0 and NP2EO) show elevated estrogenic potency. Previous studies by Jobling and Sumpter (1993) and White and colleagues (1994) also demonstrated that NP and NP2EO could stimulate the estrogen-responsive vitellogenin gene in trout hepatocytes at concentrations of 1000 nM and above. These compounds also increased breast cancer cell growth and stimulated ER transcriptional activity by transient transfections of an ERE reporter gene construct in MCF-7 breast cancer cells (White *et al.*, 1994). Interestingly, the carboxylate acid derivatives of NPEOs (NP1EC and NP2EC) tested failed to induce estrogenic activity in the ER-CALUX assay (Table 3). However, White *et al.* (1994) showed NP1EC to be slightly more potent than NP or NP2EO in the cell-based assays described above. NP1EC and NP2EC have also been shown to induce reporter gene activity in a recombinant yeast estrogen screen at concentrations above 1 mg/l (Routledge and Sumpter, 1996). It is possible that the concentrations tested in this study (max. 10 µg/l) may have been too low to elicit luciferase induction. Though 4-octylphenol demonstrated estrogenic potency in the ER-CALUX, the OP ethoxylates tested (OP 8/9EO) were not estrogenic (Table 3). To our knowledge, these OP ethoxylates have not been tested earlier for estrogenic effects.

Of the phthalate plasticizers tested in ER-CALUX, only butylbenzylphthalate (BBP) demonstrated full estrogen agonism (Figure 2). BBP has been previously shown to be estrogenic, both in stimulating breast cancer cell growth (Jobling *et al.*, 1995; Soto *et al.*, 1995) and inducing ER transcriptional activity in both transiently transfected MCF-7 cells (Jobling *et al.*, 1995) and in the recombinant yeast screen (Harris *et al.*, 1997). Importantly, the high-volume use phthalate DEHP was not estrogenic in the ER-CALUX (Figure 2) or other *in vitro* estrogenicity assays (Harris *et al*, 1997).

The commonly used chloro-s-triazine-derived herbicides triazine and atrazine, as well as the atrazine degradation products desethylatrazine, and deisopropylatrazine were not estrogenic in the ER-CALUX assay (Table 3). Accordingly, a recent study showed the failure of atrazine and simazine to elicit estrogenic effects using *in vivo* (uterine wet weight) and *in vitro* (breast cancer cell growth and ER-mediated reporter gene induction) assays (Connor *et al.* 1995).

Prediction of in vivo effects using in vitro biotransformation

Chemicals may be converted to active estrogens following *in vivo* metabolism. For some sediment-associated compounds such as PAH benzo[a]pyrene, as well as some PBDEs, it has been previously shown that metabolites are responsible for *in vitro* estrogenic activity (Charles *et al*, 2000a, Meerts *et al.*, 2000). In order to provide an even more realistic screening tool, a biotransformation step was included in the ER-CALUX assay. Methoxychlor

was chosen as a model compound because it is a known pro-estrogen that must undergo hydroxylation to produce its estrogenic metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Bulger *et al.*, 1978). Pre-incubation of methoxychlor with Aroclor-induced liver microsomes resulted in higher estrogenic activity of the metabolites in the ER-CALUX assay than the parent compound (Figure 4b). Accordingly, other studies have shown that methyoxychlor is metabolised *in vivo* or using liver microsomes to produce the active estrogenic HPTE form (reviewed by Cummings, 1997). Estradiol, on the other hand, showed weaker estrogenic metabolites following pre-incubation with induced micrsomes. This is consequent with *in vivo* studies in which the stimulatory effect of liver microsomal enzymes on steroid metabolism is paralleled by a decreased action of estradiol (reviewed by Zhu and Conney, 1998). However, chemical analysis of the extracts obtained following microsomal incubations would be useful to identify which metabolites are responsible for activation or inactivation of a (xeno-)estrogen. In a recent study, Charles and colleagues (2000b) incubated methoxychlor with Aroclor 1254-induced liver microsomes directly with transiently transfected MCF-7 cells in a recombinant ERα transactivation assay. Incubation with microsomes increased the estrogenic potency of methoxchlor in this study, and HPTE was identified by HPLC as one of three main metabolites formed. The inclusion of an incubation step with liver microsomes could provide a simple addition to a sensitive *in vitro* screen such as the ER-CALUX assay to provide more insight in the *in vivo* estrogenic potency of a suspected (xeno-)estrogen.

In conclusion, the ER-CALUX assay is a very useful tool to rapidly characterize the (anti-)estrogenic potency of mixtures of compounds in small amounts of environmental matrices, such as sediment extracts. This assay appears to be more sensitive to both estrogens and anti-estrogens than the widely used recombinant yeast screen. The value of this assay is becoming increasing apparent through its current use in ongoing large-scale baseline studies to determine the exposure and effects of (xeno-)estrogens in The Netherlands (Vethaak *et al*, 2000b). Addition of a metabolic transformation step, such as pre-incubation of test compounds with P450-induced rat liver microsomes, can provide valuable information regarding the potential estrogenic effects of metabolites of (xeno-)estrogenic compounds. Further research is underway to determine if *in vitro* estrogenic activity can predict *in vivo* events using a novel test system with transgenic zebrafish expressing the same recombinant luciferase gene as in the ER-CALUX assay (Legler *et al.*, 2000a).

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Chapter 4

Measurement of estrogenic activity in human urine and fish bile using the *in vitro* ER-CALUX reporter gene assay

submitted to *Environmental Toxicology and Chemistry*

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Abstract

Estrogens are mainly excreted from humans and animals in a conjugated, biologically inactive form. Detection of "free" estrogens in the environment suggests that these conjugates are re-converted to an active form. One possibility for this conversion is that bacteria in the environment can deconjugate estrogen metabolites through enzymatic hydrolysis. We have developed a simple method to determine if estrogen metabolites present in human urine and fish bile can be deconjugated by enzymatic hydrolysis into active estrogens. Estrogen glucuronides in human urine samples were subjected to enzymatic hydrolysis via incubation with ß-glucuronidase or live *E. coli* cells. Bile from male bream and flounder sampled from various freshwater and marine locations were also deconjugated. Deconjugated glucuronides were tested for estrogenic activity in a recently developed estrogen receptor-mediated luciferase reporter gene (ER-CALUX) assay in stably transfected T47D human breast cancer cells. Estrogen glucuronides in urine obtained from human males and females, as well as in male fish bile, were indeed converted to active forms following incubation with ß-glucuronidase or *E. coli*, indicating that bacterial hydrolysis may form a major contribution to the levels of estrogenic substances found in the environment. The highest estrogenic activity was found in deconjugated metabolites from urine of a pregnant woman, in which levels up to 3000 nmol estradiol equivalents per liter urine were found following overnight incubation of urine with *E. coli*. In fish bile, high estrogenic activity of deconjugated bile metabolites correlated with elevated levels of (xeno-)estrogens in water as well as vitellogenin induction. Measurement of (deconjugated) bile and urine metabolites could form a useful biomarker for internal dose of (xeno-)estrogens in males.

Introduction

In mammals, estrogens are eliminated by metabolic conversion to less active or inactive water-soluble metabolites that are excreted via the urine and/or faeces. Oxidative metabolism of estrogens occurs mainly via hydroxylation in the liver (reviewed by Martucci and Fishman, 1993). Conjugation of hydroxylated metabolites occurs by glucuronidation, sulfonation and/or *O*-methylation (reviewed by Zhu and Conney, 1998). Conjugation is an essential step for excretion of waste products. In human urine, estrogens are excreted mainly as estrogen glucuronide conjugates, though minute quantities of parent form 17 β-estradiol (E2), as well as the metabolites estriol (E3) and estrone (E1) are also excreted. For example, women can excrete around 2.4 µg E2, 4.6 µg E2 and 7 µg E1 daily (Aldercreutz *et al.*, 1986). Estrogens may also be excreted with the faeces, as small quantities of E2, E3 and E1 have been found in the faeces of females (Aldercreutz *et al.*, 1994). Estrogens in the bile are excreted into the intestines, where they may be broken down by intestinal bacteria, thereby forming a *de novo* source of the active parent compound (Klaassen and Watkins, 1981). Ethinylestradiol (EE2), the widely used synthetic estrogen for contraception, can be excreted in higher amounts in the unchanged form in urine and faeces. In a study in which EE2 was administered orally, a total of 18.5% of the dose was excreted as unconjugated free EE2 (Reed *et al.*, 1972). In the urine, about 64% of the dose of EE2 was conjugated, compared to

70% in the faeces (Reed *et al.*, 1972). In fish, glucuronidation is the dominant conjugation reaction for biliary excretion of steroids as well as xenobiotic compounds (Truscott, 1979, Truscott, 1983). In rainbow trout, estrogens are excreted for over 90% in the bile as E2-glucuronides (Forlin and Haux, 1985).

Despite the fact that estrogens are mostly excreted in conjugated, biologically inactive forms, many recent studies have reported the presence of parent forms of natural and synthetic estrogens in the environment (Shore *et al*, 1993; Arcand-Hoy *et al.*, 1998, Desbrow *et al.*, 1998, Belfroid et al., 1999a). It is possible that conjugated estrogens become deconjugated as they pass through the sewage process, or even before reaching the sewage treatment works. Several enzymes produced by bacteria are capable of transforming steroids. For example, β-glucuronidase is capable of hydrolysing acid glucuronides back to their primary compounds (Ralovich, 1991). This enzyme is present in various micro-organisms, such *as E. coli*, in faeces and sewage treatment works. Recent studies with the fathead minnow (*Pimephales promelas*) exposed to the conjugated E2 metabolite estradiol 3-glucuronide in the presence of laboratory simulated sewage treatment processes showed estrogenic effects (i.e. the induction of plasma vitellogenin) not seen in fish exposed to E2 3-glucuronide alone (Panter *et al.*, 1999).

The objective of this study is to develop and apply a simple method to measure the bacterial deglucuronidation of (xeno-)estrogens glucuronides present in urine and fish bile in order to determine if transformation of metabolites by bacteria can form a major source of the high levels (xeno-)estrogens both in the environment and internally in fish. Incubation of human urine and male fish bile samples with β-glucuronidase from *E. coli* and the mollusc *H. pomatia,* as well as live *E. coli* cells, was used. The estrogenic activity of deconjugated (xeno-)estrogens was quantified using the *in vitro* estrogen receptor (ER)-mediated Chemical Activated Luciferase gene eXpression (ER-CALUX) assay with stably transfected T47D breast cancer cells. To determine if estrogenic activity in deglucuronidated fish bile samples could provide an indication of internal dose of (xeno-)estrogens, comparison with induction of plasma vitellogenin in the same fish was performed. In addition, the estrogenic potency of a number of (xeno-)estrogens that may be present in urine and bile was determined using the ER-CALUX assay.

Materials and Methods

Chemicals

17β-estradiol (E2, 99%), estradiol 3(B-D-glucuronide (E2-glucuronide, 99%), estriol (E3, 99%), benzo[a]pyrene and ethanol (100%, p.a.) were purchased from Sigma Chemicals. Estrone (E1, p.a.) and 17α–estradiol (E2-17a, p.a.) were from Brunschwig. Ethinylestradiol (EE2, 98%) was purchased from Aldrich. Dimethyl sulfoxide (DMSO, 99.9%, spectrophotometric grade) was purchased from Acros.

ER-CALUX assay procedure

The ER-CALUX assay procedure and cell culture is described in detail elsewhere (Legler *et al.*, 1999). Briefly, stably transfected T47D cells were plated in clear plastic 96 well

plates (Nucleon, Denmark) at a density of 5000 cells in 0.1 ml DMEM-F12 without phenol red supplemented with 5% dextran-coated charcoal treated fetal bovine serum (DCC-FBS) per well. Following 24 hours incubation, medium was renewed and the cells incubated for another 24 hours. The medium was then removed and the cells were dosed in triplicate by addition of the dosing medium containing the chemical or extract to be tested dissolved in ethanol or DMSO (max. 0.2%). Control wells, solvent control wells and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. Following 24 hours treatment, medium was removed and cells were lysed in 50 µl triton-lysis buffer, pH 7.8 (containing 1% triton X-100, 25 mM glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT) for a minimum of 1 hour with gentle shaking at 4°C. A sample of 25 µl lysate was then transferred to a black 96 well plate (Costar) and 25 µl of luciferin solution (Luclite, Packard) was added per well. Luciferase activity was assayed in a scintillation counter (Top Count, Hewlett-Packard) for 0.1 minute per well.

Extraction of estrogen conjugates from urine

For all extraction procedures, glassware was pre-rinsed with ethyl acetate (p.a., Merck).

First morning urine was collected from males and females and aliquoted in 2 ml portions over 4 glass test tubes. Concentrated hydrochloric acid (HCl) was added with a pasteur pipette, one drop per ml urine, and the tubes vortexed. Ethyl acetate (2 ml) was added to each test tube, the tubes vortexed for one minute, and centrifuged 5 minutes at 3800 rpm. The ethyl acetate fraction was removed with a pasteur pipette and transferred to a clean test tube. Because of protein formation in between the water/solvent phases, 500 µl isopropanol was added once after centrifugation. The extraction was repeated a total of 3 times, after which the collected ethyl acetate extract was carefully evaporated to a small drop under a gentle N2 gas flow at 37°C. The extract was transferred to a glass conical vial and the test tube rinsed 3 times with ethyl acetate, and transferred to the conical vial. The remaining ethyl acetate was evaporated at 37 °C under N2 gas. The extract was then taken up in 100 µl ethanol.

Deconjugation of urinary and biliary estrogen conjugates

As a positive control of the deconjugation reactions in all experiments, 1 nmol 17 β-estradiol 3(B)-D-glucuronide per ml urine was included in the procedures described below.

i) Incubation of urine with ß-glucuronidase preparations

ß-glucuronidase purified from *E. coli* or ß-glucuronidase/arylsulfatase from *H. pomatia* (Sigma) were compared. For reactions with ß-glucuronidase purified from *E. coli*, 885 µl distilled water was added to a test tube, followed by 500 µl of a 75 mM potassium phosphate buffer ((KH2P04), pH 6.8 with 1% (w/v) bovine serum albumin). Urine extract (15 µl) then added to the tube. The solutions were mixed and equilibrated at 37°C. 100 µl ß-glucuronidase (400 U/ml in cold KH2P04 buffer) was added, mixed by inversion, and incubated for 30 minutes, 3 hours or 18 hours at 37°C. For incubations with ß-glucuronidase/arylsulfatase enzymes purified from *H. pomatia*, the incubation mixture was

composed of 700 µl buffer (100 mM sodium acetate buffer (NaAc), pH5.0), 685 µl distilled water, 15 µl urine extract, and 100 µl enzyme (400 U/ml NaAc buffer).

In addition to deconjugation using urine extracts, pure urine was also used. First morning urine was collected from a man, woman and pregnant woman. Distilled water (1 ml) was used as a control. For reactions with ß-glucuronidase purified from *E. coli*, 1 ml urine was mixed with 555 µl KH2P04 buffer and 40 U ß-glucuronidase. For reactions with ß-glucuronidase/arylsulfatase enzymes purified from *H. pomatia*, 1 ml urine was mixed with 1 ml NaAc buffer and 40 U enzyme. Incubations with pure urine were carried out for 18 hours at 37°C.

ii) Incubation of urine with live E. coli culture

Urine estrogen conjugates in pure urine from male, female, 6-month pregnant female and control (water only) were also deconjugated using wild type *E. coli* cells. *E. coli* cells were cultured overnight in a 37°C water bath with gentle shaking in tubes containing 10 ml growth medium (10 g/l bacto-tryptone, 5 g/l yeast extract and 10 g/l NaCl). First morning urine (1 ml) was added to duplicate tubes, followed by 1 ml growth medium. A 10 µl sample of the overnight *E. coli* culture (OD 1) was transferred to the tubes, and left overnight to grow in a 37°C water bath.

iii) Fish bile incubation with β-glucuronidase preparations

The freshwater and marine sampling locations for bream (*Abramis brama*) and flounder (*Platichthys flesus*) in The Netherlands are described elsewhere (Vethaak *et al.*, 2000c). Bile samples were frozen and stored at -20°C. Bile samples were thawed on ice, and samples of 100 µl bile were transferred to glass test tubes. Sodium acetate-3H20 buffer (100 mM, pH 5.0 at 37°C) was added at a volume of 700 µl. Distilled water (600 µl) and 100 µl of 400 U/ml β-glucuronidase/arylsulfatase (from *H. pomatia*) was added. Tubes were incubated overnight (17-18 hrs) in a 37°C water bath with gentle shaking.

Extraction of deconjugation products

Following incubation of urine (extracts) and fish bile with β-glucuronidase enzyme preparations or *E. coli* cells, deconjugations products were extracted with ethyl acetate. For samples with *E. coli* cells, tubes were first centrifuged at 3800 rpm for 20 minutes to pellet the cells. The supernatant was then transferred to a fresh tube. For the extraction of urine and bile, 2 drops of HCl (1 N) were added to each tube and vortexed. Ethyl acetate (2 ml) was then added, and the samples vortexed for 1 minute and centrifuged at 3800 rpm for 5 minutes. The overlying ethyl acetate fraction was transferred to a clean tube, and the extraction procedure repeated a total of three times. The collected ethyl acetate extract at 37°C was carefully evaporated to a small drop under a gentle N2 gas flow. The extract was transferred to a glass conical vial and the test tube rinsed 3 times with ethyl acetate, and transferred to the conical vial. The remaining ethyl acetate was evaporated at 37 °C under N2 gas. The extract was then taken up in 50 µl DMSO and tested in the ER-CALUX assay as described above.

Extraction efficiency

The efficiency of extracting deconjugation products was determined using ^{3}H-labelled estradiol (^{3}H-E2). The radiolabeled E2 was added to 2 ml of buffer reagent from the reactions with ß-glucuronidase, or 2 ml of *E. coli* growth medium in glass tubes. The samples were vortexed and left to stand for 15 minutes. The extraction was carried out as described above using four extraction solvents: dichlormethane, ethyl acetate, diisopropylether and hexane:dietylether (97:3). After each extraction step (three in total), the extract was transferred to a plastic counting tube. To each tube, 9 ml of counting fluid (Ultima Gold, Packard) was added and the tubes are shaken well. The amount of ^{3}H was measured in a liquid scintillation analyzer (Packard).

Estrogenic activity in environmental samples

Samples of water, particulate matter, sediment and biota (fish and mussels) were sampled from the same location as the fish sampled for the bile studies. The extraction of these samples is described elsewhere (Vethaak et al., 2000c). These extracts were dissolved in DMSO and tested in the ER-CALUX assay as described above.

Vitellogenin induction

Vitellogenin (VTG) was measured in plasma in the same male bream and flounder in which bile estrogenic activity was determined. VTG was measured using a 96-wells competitive ELISA method adapted from Smeets *et al* (1999). The primary antibody, raised against goldfish by the Michigan State University, showed good cross-reactivity with bream VTG.

Data analysis

For quantification in the ER-CALUX assay, data shown are representative of a minimum of three independent assays. Extraction with urine and bile were carried out in a minimum of two independent experiments. For quantification of the estrogenic potency of an environmental sample, urine or bile extract, the response of the extract is interpolated in a dose-response curve of the standard E2. For this the curve-fitter of SlideWrite 4.0 was used (cumulative fit). The R of the fit of the standard curve was above 0.98. The extracts were diluted so that the response used for interpolation was between the signal of 1 and 6 pM (linear portion of curve) only. The estrogenic potency of the extract is expressed as EEQ (estradiol equivalents) per volume of material.

Results and Discussion

Estrogenic potency of natural, synthetic and xeno-estrogens in ER-CALUX assay

In order to determine the sensitivity of the ER-CALUX assay to natural, synthetic and xenobiotic estrogens that may be present in urine, we exposed cells to parent compounds (estradiol, E2), metabolites (17α-estradiol (E2-17α), estrone (E1) and estriol (E3)) and one E2-glucuronide conjugate, as well as ethinylestradiol (EE2) and benzo(a)pyrene (BAP). Exposure of T47D.Luc cells to E2 for 24 hours resulted in a detection limit of 0.5 pM and an EC50 of 6 pM (Figure 1). Maximum induction at 30 pM E2 relative to control was about 100 fold. E1 and 17α-E2 were about 5-500x less potent than E2 (Figure 1), with an EC50 of 27 and 530 pM, respectively. Interestingly, E3 was as potent in the ER-CALUX assay as E2 (Figure 1), with an EC50 of 6 pM. E3 has been previously shown to have about 10 times less binding affinity to rat ER (Blair *et al.*, 2000), as well as 10 times less ER transactivational activity (Melamed *et al.*, 1997) than E2. The mechanism underlying the equal potency of E3 and E2 in the ER-CALUX assay are unclear, though tissue specific effects of E3 are possible, as E3 was also shown to be a full agonist in breast cancer cell proliferation (Soto *et al.*, 1995). The synthetic estrogen EE2 was slightly more potent than E2, with an EC50 of 5 pM (Figure 1). The higher potency of EE2 compared to E2 is due its higher binding affinity to the ER (Blair *et al.*, 2000), as well as its stability and resistance to metabolisation (Guengerich, 1990). T47D.Luc cells possess minimal intrinsic deconjugation activity, as exposure to increasing concentrations of β-estradiol 3-(B-D)-glucuronide (E2-gluc) did not result in increased luciferase activity (Figure 1).

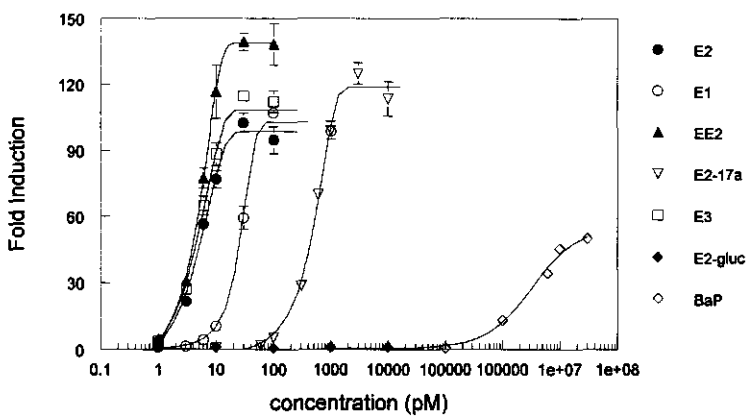

Figure 1: Estrogenic activity (fold induction relative to solvent control) of various estrogens, including natural estrogens (estradiol (E2); estrone (E1) 17α-estradiol (E2-17a) and estriol (E3)), estrogen metabolites (β-estradiol 3-(B-D)-glucuronide (E2-gluc)), synthetic estrogens (ethynylestradiol (EE2)) as well as benzo[a]pyrene (BaP) in the ER-CALUX assay.

In addition to the natural and synthetic estrogens, BaP was tested for estrogenic potency because its hydroxylated metabolites are widely detected in human urine (reviewed by Angerer *et al.*, 1997) and fish bile (Leadly *et al.*, 1999 and references therein) as a biomarker of exposure to aromatic hydrocarbons. BaP demonstrated weak estrogenic activity in the ER-CALUX assay, with an EC50 of 3200 nM (Figure 1). Charles and colleagues (2000) also recently demonstrated the estrogenic activity of BaP in an ER-α mediated reporter gene assay, and provided evidence that estrogenic activity was predominantly produced by hydroxylated metabolites of BaP.

Enzymatic deconjugation of estrogen glucuronides in urine

Extraction of deconjugated estrogens was performed with ethyl acetate because this solvent resulted in the highest recovery (98% recovery, data not shown) of the solvents tested using ^{3}H-E2 spiked reaction buffer. Enzymatic deconjugation of estrogen glucuronides in (extracts of) male and female urine samples resulted in increased estrogenic activity as measured in the ER-CALUX assay (Table 1). These results indicate that inactive metabolites in urine can be readily converted to biologically active estrogens by bacterial enzymes. Urine spiked with β-estradiol 3-(B-D)-glucuronide (E2-gluc) was efficiently converted to active E2 by β-glucuronidase from both *E. coli* and *H. pomatia* (Table 1). Of the 1 nmol/ml E2-gluc spiked urine, 1 to 1.4 nmol/ml EEQ was found following 18 hour incubation with both enzyme preparations (Table 1), indicating complete enzyme hydrolysis. In general, longer incubation times with β-glucuronidase preparations (up to 18 hours) increased the activation factor (Table 1). Higher (about 2-3 times) deglucuronidation of estrogen glucuronides was found pure urine samples than in urine extracts when incubated for 18 hours (Table 1). This difference is likely due to the loss of glucuronides during the first extraction step of urine. Although *H. pomatia* has both glucuronidase and sulfatase activity so estrogen sulfates may be also hydrolysed, little difference was found in the estrogenic activity in urine metabolites conjugated with β-glucuronidase purified from *E. coli* and *H. pomatia*. Previous studies have also shown little difference in these 2 enzyme preparations to hydrolyse estriol glucuronides in urine (Graef *et al*, 1977). Interestingly, in both pure urine and urine extracts, the difference in deconjugated estrogens between female male and urine is about a factor of 4 (Table 1). This corresponds to reports of estrone excreted by women (3-20 ug/person/day) and men (5 ug/person/day) (Gower, 1975).

Hydrolysis of estrogen glucuronides using E. coli cells

Incubation of pure male and female urine with *E. coli* cells resulted in an efficient conversion of estrogen conjugates to active estrogens that could be easily measured in the ER-CALUX (Table 2). Extraction efficiency of ^{3}H-E2 from *E. coli* growth medium was about 98% using ethyl acetate (data not shown). Urine sampled from the pregnant female contained the most estrogen conjugates, as conversion by *E. coli* resulted in extremely high estradiol equivalents (2800 nmol EEQ/l urine), about 150 times more than a non-pregnant female. During pregnancy, estriol is the most abundant estrogen, and E3 levels rise to about ten times that of estradiol and estrone (De Hertogh *et al.*, 1975). In this study, E3 was as active as E2 in

the ER-CALUX assay (Figure 1). Assuming a production of 2 liters of urine per day, the EEQ level found in the pregnant woman in this study would be the equivalent to about 5 µmol estrogens/day. Accordingly, Fostis (1987) reported excretion of up to 10 µmol/day of estrogens by pregnant women. These results provide clear evidence that *E. coli* can convert metabolites present in urine into biologically active estrogens. This efficient conversion of glucuronides to active estrogens forms a major source of estrogens in the aquatic environment. In a recent study in The Netherlands, elevated estrogenic activity was measured using the ER-CALUX assay in influent and effluent samples from treatment plants receiving domestic wastewater (Murk *et al.*, 2000). The highest contribution to the estrogenic activity measured in these samples was the natural estrogens E2 and E1, as well as EE2 (Murk *et al.*, 2000).

Enzymatic hydrolysis of estrogen glucuronides in fish bile

Bile samples from male bream and flounder collected from 5 locations within The Netherlands contained metabolites that could be converted to active estrogens via enzymatic hydrolysis (Table 3). Activation factors following deglucuronidation of bile samples ranged from 17-590 (data not shown). Male bream sampled from a small freshwater river (The Dommel) showed high levels of biliary (xeno-)estrogens (up to 8700 pmol EEQ/ml) in comparison to the freshwater reference site Vrouwenzand (up to 65 pmol EEQ/ml) (Table 3). Accordingly, elevated EEQs were found in the surface water of this river (8.5 pmol EEQ/l), as well as in freshwater mussels (145 pmol EEQ/l) (Table 3). Additional evidence of exposure to (xeno-)estrogens at this location was found in the extremely high plasma vitellogenin levels in the same bream (Table 3). Recent studies have shown that the wastewater treatment plant effluent released in this river contains high levels of compounds that are estrogenic in the ER-CALUX as well as a novel *in vivo* assay using estrogen-sensitive transgenic zebrafish (*Legler et al.*, 2000d). In partial life cycle studies with zebrafish, chronic exposure to this effluent has been found to influence sexual differentiation (Bulder *et al*, 2000). In addition, wild populations of bream sampled from this river showed increased number of hermaphroditic fish compared to other locations in The Netherlands (Vethaak *et al*, 2000b and 2000c).

Interestingly, bile sampled from male flounder showed relatively little difference in estrogenic activity in between reference areas (Eastern Scheldt) and relatively polluted areas (Amsterdam) (Table 3). Plasma vitellogenin levels in these flounder were also not elevated (Table 3). As the flounder is a benthic species, it is likely that it is mainly exposed to (xeno-)estrogens through contact with the sediment. Estrogenic activity in the sediment was low in all three locations (Table 3), suggesting that the flounder were not exposed to elevated levels of (xeno-)estrogens. In addition, flounder may not be as sensitive to estrogenic compounds as other fish species, as laboratory exposure of flounder to EE2 showed a threshold value for vitellogenin induction about 10 times higher than rainbow trout (Allen *et al.*, 1999).

Table 1: Estrogenic activity of deglucuronidated estrogen glucuronides in male and female urine (nmol estradiol equivalents EEQ/L urine). β-glucuronidase (β-glucuron) purified from *H. pomatia* (also containing arylsulfatase activity) and *E. coli* was incubated with pure urine or urine extracts for various incubation periods (inc. time).

Matrix	Source β-glucuron.	Inc. time (hours)	nmol EEQ/L urine	Activation Factor[a]
Urine (pure)				
Male	***H. pomatia***	0 (control)	0.2	
		18	16	77
	E. coli	18	21	101
Female	*H. pomatia*	0 (control)	0.9	
		3	26	28
		18	71	77
	E. coli	3	82	89
		18	69	75
Male, spiked[b] E2-gluc.	*H. pomatia*	0 (control)	22	
		3	299	14
		18	1042	47
	E. coli	3	1109	50
		18	1422	65
Urine (extract)				
Male	*H. pomatia*	0 (control)	0.2	
		3	4.0	20
		18	6.6	33
Female	*H. pomatia*	0 (control)	0.6	
		3	22	37
		18	25	42
	E. coli	3	22	37
		18	26	43
Male, spiked E2-gluc.	*H. pomatia*	0 (control)	0.9	
		0.5	347	386
		3	1753	1948
		18	1082	1202

[a] Activation factor represents the factor increase in estrogenic activity relative to control.
[b] Male urine was spiked with 1 nmol β-estradiol 3-(B-D)-glucuronide (E2-gluc)/ml urine.

Table 2: Estrogenic activity of deglucuronidated estrogen glucuronides in male and female urine (nmol estradiol equivalents EEQ/L urine). Pure urine was incubated overnight with live *E. coli* cells and hydrolysed estrogens were extracted and tested in the ER-CALUX assay.

Matrix	Conversion	nmol EEQ/L urine	Activation Factor[a]
Urine (pure)			
Male	- *E. coli*	2	
	+ *E. coli*	20	10
Female	- *E. coli*	8	
	+ *E. coli*	19	2
Pregnant Female	- *E. coli*	34	
	+ *E. coli*	2825	83
male, spiked[b] E2-gluc.	- *E. coli*	2	
	+ *E. coli*	2635	1318

[a]Activation factor represents the factor increase in estrogenic activity relative to control.

[b] Male urine was spiked with 1 nmol β-estradiol 3-(B-D)-glucuronide (E2-gluc)/ml urine.

Comparison of the results of biliary (xeno-)estrogenic activity and VTG induction in male bream in a polluted system suggests that measurement of estrogenic activity in bile may provide a useful indication of internal dose of (xeno-)estrogens for this fish species. Because the sample size in this study was small, results from a previous study were also analysed. Male bream were sampled from 8 different locations in the spring of 1999 in the Netherlands, and bile estrogenic activity measured in the ER-CALUX assay following deglucuronidation. A good correlation (coefficient of determination = 0.81) was found between bile EEQs and plasma vitellogenin induction in these fish (Figure 2). Of course, chemical analysis of bile samples is necessary to determine the identity of (xeno-)estrogens that may contribute to internal levels. In addition to estrogen glucuronides, BaP and nonylphenol are two examples of xeno-estrogens that are present in fish bile mainly as glucuronides (James *et al.*, 1991; Arukwe *et al.*, 2000). In this study, BaP and in a previous study, nonylphenol (Legler *et al.*, 1999) were demonstrated to be estrogenic in the ER-CALUX assay.

Table 3: Estrogenic activity of deglucuronidated estrogen glucuronides in male fish bile (pmol estradiol equivalents EEQ/ml bile). Deglucuronidated (xeno-)estrogens were extracted and tested in the ER-CALUX assay. EEQs in extracts of water (pmol/l), particulate matter and sediment (pmol/g) and biota (fish and mussels) (pmol/kg) taken from the same location. Vitellogenin (VTG, ng/ml) was measured in plasma of same fish.

		Estradiol equivalents (EEQ)						
Fish #	Location	Bile[a]	water	part. mat.	sed.	fish	mussels	VTG
Bream								
1	Vrouwen-	65	0.0	n.m.[*]	0.2	2.3	34.2[c]	51
2	zand	58						143
4		34						141
5		51						54
3	Dommel	2265	8.5[b]	n.m.	1.0	11.8	145.4[c]	26E6
4		8670						25E6
9		3865						15E6
13		1150						3.0E6
Flounder								
37	Eastern	92	0.1	0.1	0.1	1.8	0.9[d]	511
9	Scheldt	61						<d.l.[**]
27		281						<d.l.
3		38						1095
1	Amsterdam	166	0.6	0.1	0.0	21.6	23.9[c]	888
9	North Sea	164						1287
16	Canal	115						242
34		59						<d.l.
4	Den Oever	328	0.0	0.1	0.0	1.5	n.m.	330
16		54						<d.l.
14		76						<d.l.
21		56						<d.l.

[a]β-glucuronidase purified from *H. pomatia* was incubated with bile for 18 hours.
[b]sample taken from receiving waters of waste water treatment plant effluent (Legler *et al.*, 2000d)
[c]freshwater mussels, n=400
[d]saltwater mussels, n=200
[*]n.m.: not measured
[**]<d.l.: below detection limit

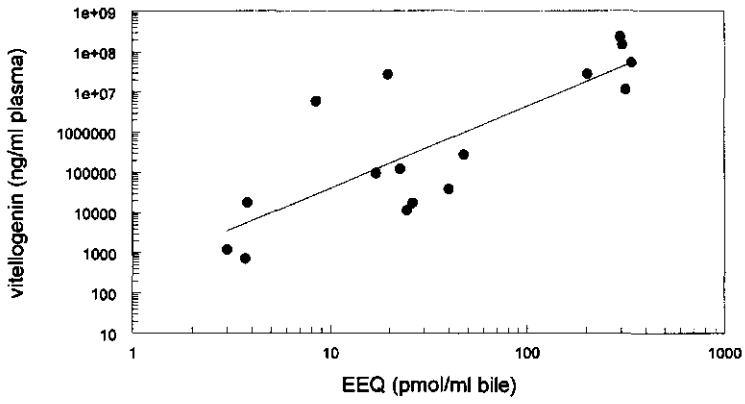

Figure 2: Estrogenic activity (EEQ, pmol/ml) in bile and vitellogenin (ng/ml plasma) in male bream from 8 different locations in The Netherlands.

In conclusion, we have developed a method to rapidly test if estrogenic metabolites present in small samples of urine and bile can be transformed to active estrogens through enzymatic hydrolysis using a rapid and sensitive *in vitro* reporter gene assay. Indeed, enzymatic hydrolysis by *E. coli* is very effective in transforming conjugated metabolites to active parent compounds. We propose that this effective transformation of metabolites by bacteria can form a major source of the high levels of natural and xenobiotic estrogens found in environmental compartments. Though chemical validation is needed, the use of the ER-CALUX assay with deconjugated bile and urine estrogen metabolites may form a promising biomarker of internal exposure.

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Abstract

Adverse trends in the reproductive health of male fish, including testis abnormalities and intersex gonads, have been increasingly reported over recent years. These effects have been associated with the exposure of fish to natural, synthetic and xenobiotic estrogens present in the aquatic environment. A novel *in vivo* test system using transgenic zebrafish has been developed to rapidly determine the effects of estrogenic chemicals on critical life stages and sensitive target organs in the fish. In the transgenic zebrafish, an estrogen binding sequence linked to a TATA box and luciferase reporter gene was stably introduced. Binding of a substance to endogenous estrogen receptors (ER) and the subsequent transactivation of the ER result in luciferase gene induction that is easily measured in tissue lysates. Exposure to estradiol (E2) during juvenile stages of the transgenic zebrafish revealed the period of gonad differentiation to be the most responsive early life stage. In adult male transgenic zebrafish, the testis was the most sensitive and responsive target tissue to estrogens. Partial sequences of zebrafish estrogen receptor subtypes α and β were cloned for the first time and were found to be differentially expressed in developing fish and tissues of adult male zebrafish. The transgenic zebrafish assay is a promising new tool to rapidly determine the estrogenic potency of chemicals *in vivo*.

Introduction

Endocrine disruption is an issue that has raised public concern and is on the political and research agenda of governments worldwide. Reports of chemicals in the environment that can mimic the actions of endogenous estrogens, thereby disturbing normal endocrine functions and causing male reproductive dysfunction in humans and wildlife (reviewed in Colburn *et al.*, 1993; Golden *et al.*, 1998, Sumpter, 1998), are increasing. In wild fish populations, intersex (the simultaneous presence of both male and female gonadal characteristics) and testis abnormalities have been found in a high proportion of male fish sampled in rivers, estuaries and coastal waters (Jobling *et al.*, 1998, Lye *et al.*, 1997, Allen *et al.*, 1999). These feminizing effects have been associated with exposure to environmental levels of natural, synthetic and xenobiotic chemicals (xeno-estrogens) in the aquatic environment. Natural estrogens include the female hormones estradiol, estrone and estriol. Synthetic estrogens are pharmaceutical chemicals designed to mimic the action of natural estrogens, such as the birth control pill component ethinylestradiol as well as diethylstilbestrol. Xeno-estrogens can be defined as environmental and industrial pollutants that are not designed to be used as estrogens, but nevertheless can evoke effects via the estrogen receptor signal transduction pathway. Laboratory exposure of male fish to (xeno-)estrogens has resulted in the synthesis of high levels of the estrogen-inducible yolk precursor protein vitellogenin (VTG) as well as inhibited testicular growth, testis abnormalities and formation of intersex gonads (reviewed in Tyler *et al.*, 1998).

Regulations aimed at determining a substance's potential to disrupt endocrine systems have proven to be extremely difficult because estrogenic substances often have very different chemical structures, hampering their analysis and risk assessment on a structural basis. Tests

to determine estrogenic effects on laboratory animals are available but are laborious, time-consuming, costly, and may require large amounts of animals. As an alternative, simpler screening methods such as *in vitro* reporter gene assays have been developed, allowing large-scale screening of chemicals (reviewed in Zacharewski, 1997). These assays make use of the fact that the receptor for estrogens is a transcription factor that induces transcription of target genes after binding to specific DNA sequences in their promoter. However, major drawbacks of such cell lines are, compared to *in vivo* measurements in animals, that important aspects of *in vivo* functioning such as metabolic conversion and breakdown can be missed. Moreover, no assessment can be made of the vulnerability of critical life stages, such as developing embryos, to the hormonal disrupting compounds.

With this in mind, we have developed a novel test system for (xeno-) estrogens using zebrafish in which an estrogen responsive reporter gene has been stably introduced. Using transgenic reporter zebrafish, the direct effects of estrogenic chemicals on estrogen-sensitive tissues can be readily determined during various stages of sexual development. Because of the large number and rapid development of offspring, transgenic zebrafish can combine the advantages of *in vitro* and *in vivo* systems to provide a rapid and simple *in vivo* model to screen for hormonally active substances. In addition, zebrafish genetics and early development have been widely studied (Haffter and Nusslein-Volhard, 1996) and it is a recommended freshwater fish species for toxicity testing (OECD, 1992). Using these transgenic fish, we show reporter gene induction *in vivo* by (xeno-) estrogens following short term exposure, demonstrating the presence of highly responsive estrogen receptors in sexually differentiating juvenile fish. Of the wide range of organs tested in adult male fish, the reproductive organs appear to be the main target tissue for estrogens.

Experimental section

Generation of transgenic zebrafish

Zebrafish were maintained at 27°C on a 14 hours light/10 hours dark photoperiod and were fed brine shrimp *Artemia salinas* four times daily. To generate transgenic zebrafish, zebrafish embryos were microinjected with 15 pg of the supercoiled DNA construct pEREtata-Luc (Legler et al., 1999) prior to first cleavage essentially according to Stuart *et al*, 1988. In total, about 1600 embryos were injected. At 24 hours post fertilization (h.p.f.), 940 (56%) of embryos survived and were individually tested for luciferase expression by immersion in 200 µl of a non-toxic luciferin substrate solution (20 mM tricine, 1 mM $(MgCO)_4MgOH_2$-$5H_20$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 270 µM coenzyme A, 470 µM luciferin, 530 µM ATP, pH 7.8)) in 96-well plates. Embryos were assayed for luciferase activity in a scintillation counter (Top Count, Packard). Embryos with luciferase expression (about 500 embryos or 55% of the survivors) were selected and reared to sexual maturity (approx. 3 months). Potential founder transgenic fish were crossed with wild type zebrafish. F_1 offspring at 24-48 h.p.f. were pooled in eppendorf tubes (about 60 embryos per tube) and lysed overnight at 55°C in 500 µl lysis buffer containing 100 mM Tris.HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 µg/ml proteinase K. Genomic DNA was extracted

each of dATP, dCTP, dGTP and dTTP (Gibco B.R.L.) and 1.5 units of TAQ polymerase (Eurogentec). Primers for zfERα, zfER-β and GAPDH were used a concentration of 20 pmol per reaction. PCR reactions were carried out in a Biometra PCR using 35 cycles of amplification for each reaction. The following conditions were used: denaturation at 95°C for 5 min., annealing at 53°C (zfERα), 68 °C (zfERβ) or 58°C (GAPDH) and extention at 72°C for 1 min. As a positive control, a sample of 10000 copies of zebrafish liver cDNA was included in the amplification reaction. No PCR products were found in RNA or water controls.

Immunohistochemistry

Adult male transgenic and wild type fish were fixed in 4% paraformaldehyde containing 10% EDTA and embedded in paraffin. Immunohistochemistry was performed on 5 µm sections following antigen retrieval by heating sections four times for 5 minutes in 10 mM citrate buffer, pH 6. Following blocking with 20% swine serum, sections were incubated with a 1:200 dilution of an affinity purified rabbit polyclonal anti-luciferase (Promega), followed by avidin-conjugated swine anti-rabbit Ig, and avidin-peroxidase with a biotin bridge. Reactivity was visualized using diaminobenzidine + H_2O_2 (Sigma).

Results and Discussion

We developed transgenic zebrafish stably expressing pEREtata-Luc an estrogen responsive luciferase reporter gene regulated by three estrogen response elements (ERE) upstream from a TATA box. The pEREtata-Luc construct and activation by estradiol *in vitro* have been described elsewhere (Legler *et al.*, 1999). Since enhancer and promoter regions other than the ERE are absent in this construct, it very specifically responds to estrogen receptor (ER) activation. Compounds activating endogenous estrogen receptors in target cells lead to receptor binding to the EREs and consequently activate transcription of the luciferase gene. Following short term exposure of transgenic zebrafish to (xeno-) estrogens, the production of luciferase protein can be easily assayed by preparing tissue lysates, and measuring light activity following addition of the enzyme substrate luciferin.

Estrogen receptor gene activity is developmentally regulated in transgenic zebrafish

Expression of luciferase in transgenic zebrafish was determined in developing life stages following 48 hours exposure in water to 1000 nM E2 (Fig. 1a). Range finding toxicity tests carried out prior to these experiments under the same exposure periods established that this concentration did not cause acute toxicity (data not shown). In embryos less than 28 days post fertilization (d.p.f.), luciferase activity could be easily assayed in lysates of pooled embryos. In fry between 28 and 35 d.p.f. of approximately 1 cm length, luciferase activity was assayed in lysates of individual embryos. Luciferase induction increased with age and stage of gonad differentiation, ranging from 4-fold in 1 day old embryos (1 d.p.f.) to 100 to 300-fold induction in 35 d.p.f. juvenile transgenic fry relative to vehicle-exposed controls

(Fig. 1a). Gonad differentiation in the zebrafish commences at about 2 weeks of age in zebrafish (Takahashi, 1977). Clear evidence of female ovarian and male testicular gonad differentiation has been observed in fry at about 28 d.p.f. (Wester, unpublished results). Our results indicate that transgene induction correlates with the sexual development of the zebrafish.

As transgene expression is regulated by the ER, we determined the timing of expression and distribution of the zebrafish ER (zfER). We isolated specific fragments of ER subtypes α and β in zebrafish ovarian cDNA and designed specific primers for semi-quantitative PCR (see Experimental Section). It should be noted that the PCR reactions with primers for zfER-β were more efficient than with the zfER-α primers (Fig. 1b, control), indicating that the absolute levels of zfER-α mRNA may be underestimated. zfER-α mRNA was detected at all stages tested in the developing embryo and fry, though the expression is highest late in sexual differentiating stages (35 d.p.f.) (Fig. 1b). zfER-β mRNA was also detected at all stages, though was low at 14 d.p.f., the stage of the onset of gonad differentiation. From 28 to 35 d.p.f., ERβ mRNA expression increased, coinciding with high luciferase induction (Fig. 1a). Our results suggest that estrogen receptors are very active during sexual development. Therefore, the period of gonad differentiation may be very sensitive to disruption by estrogenic compounds.

Ligand sensitivity of reporter gene induction in juvenile fish

In order to determine the sensitivity of juvenile fish to E2, dose-response studies were carried out with transgenic 35 d.p.f. fry (Fig. 2a). A nominal concentration of 0.1 nM E2 resulted in 7 ± 4-fold induction following 96 hours exposure (Fig. 2a), indicating that the juvenile fish undergoing sexual differentiation may be sensitive to E2. The concentration of 0.1 nM E2 is a realistic concentration that may be encountered in aquatic systems in the environment (Desbrow *et al.*, 1998). Some natural, synthetic and xenobiotic estrogens were also tested for their potential to induce luciferase in juvenile transgenic zebrafish. Following 48 hour exposure to a nominal concentration of 1000 nM, the natural estrogens 17α-estradiol and estrone, as well as the synthetic estrogens diethylstilbestrol and ethinylestradiol, induced luciferase at levels ranging from 70 to 300 times that of non-exposed controls (Fig. 2b). Importantly, the principle DDT isomer o,p'DDT, a known environmental estrogen in mammals (Bitman *et al.*, 1969) and fish (Denison *et al.*, 1981), also induced luciferase activity to a level around 40 times that of the solvent control (Fig. 2b). It should be noted that relatively high variation was found in the response of individual transgenic fish to estrogens. The outbred genetic background of these heterozygous transgenic fish may contribute to the variation in estrogenic response observed since differences in genetic background may greatly influence the response to estrogens (Spearow *et al.*, 1999). In addition, both males and females undergoing gonad differentiation were assayed together at this stage, as their phenotypic sex is not yet apparent. It was found that males and females respond differently to exogenous estrogens (see below).

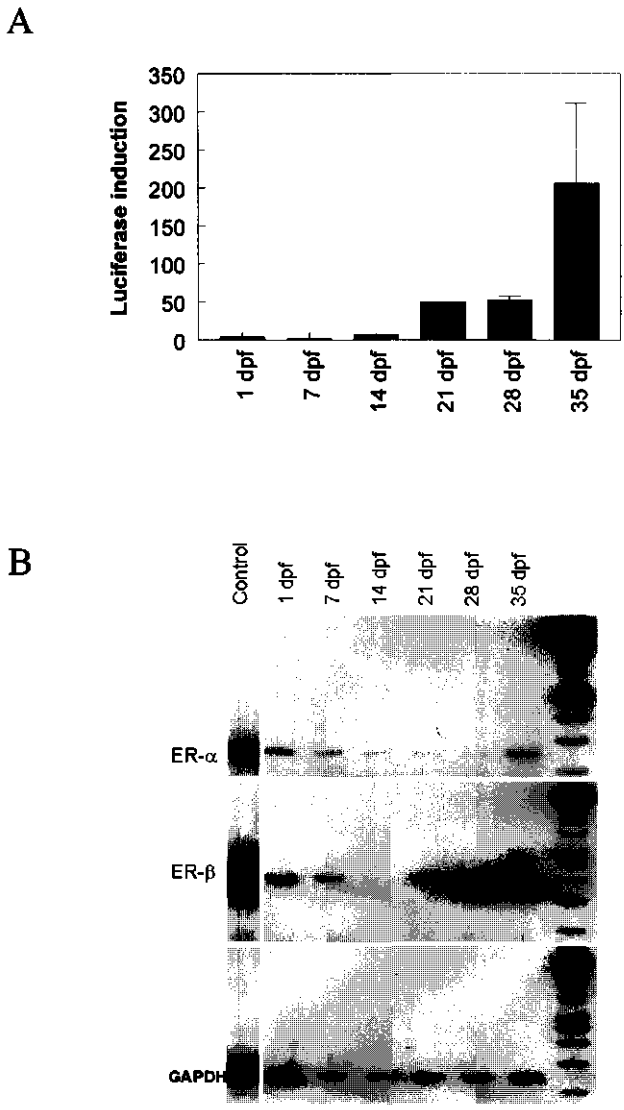


Figure 1: (A) Luciferase activity in developing life stages of transgenic zebrafish exposed for 48 hours to 1000 nM 17β-estradiol (fold induction in light units/μg protein relative to vehicle exposed controls) and (B) stage-related expression of zebrafish estrogen receptor type ER-α and ER-β mRNA in non-exposed developing life stages (dpf=days post fertilization). "Control" lane shows PCR product from liver cDNA (10000 copies) amplified under the same conditions. In Figure 1 (A), age given indicates age at initiation of exposure. Bars show the luciferase induction in pools of embryos from 1 to 21 dpf: 1 dpf: n=20; 7 dpf: n= 10, 14 dpf: n=10; 21 dpf: n=5. For embryos of 28 and 35 dpf, bars show mean luciferase induction in individual fry (n=4); error bars show standard error of the mean.

Estrogen reporter gene induction is highly tissue specific

To determine the tissue distribution of transgene expression, we studied the response of adult, sexually mature, male transgenic zebrafish to estrogen treatment (Fig. 3a). Surprisingly, dramatically high transgene expression of up to 1000-fold was observed in the testis following 48 hours exposure to 1000 nM E2 (Fig. 3a). In addition, luciferase activity was also prominent in liver (30-fold) and slightly induced in the scales (4-fold) and muscle (3.6-fold). Slight or no significant luciferase induction was found in heart, brain, eye or bone tissue. In control males exposed to vehicle (DMSO or ethanol) alone, luciferase was not induced, indicating extremely low levels of endogenous estrogens. In control adult transgenic females of 3-6 months of age, however, background levels of luciferase in liver and ovaries were high, reaching approximately the same level as liver in E2 exposed males. High background levels of luciferase in transgenic females are likely due to high levels of circulating endogenous estrogens. Exposure to E2 in adult females did not result in luciferase induction above this elevated background level (data not shown).

Analysis of the zfER mRNA expression pattern in various tissues of the adult male zebrafish confirmed the presence of both zfER-α and ER-β (Fig. 3b) in tissues showing high luciferase induction such as testis and liver (Fig. 3a). Tissues that did not show high luciferase induction such as brain, bone, digestive tract and eyes, however, expressed varying amounts of both receptors (Fig. 3b). Differences in the expression of the internal control GAPDH do not reflect differences in the amount of tissue amplified per reaction. GAPDH is differentially expressed in various organs, with lower expression in the brain. We therefore did not correct for GAPDH variation throughout the samples. The differences in ER mRNA expression and activation of luciferase between various tissues may be explained by differences in levels of receptor protein or co-activators necessary for ER-mediated transcriptional activation. It is also possible that the luciferase response to E2 in some tissues was limited due to the tissue-specific bioavailability of the ligand, perhaps partly due to the short exposure period.

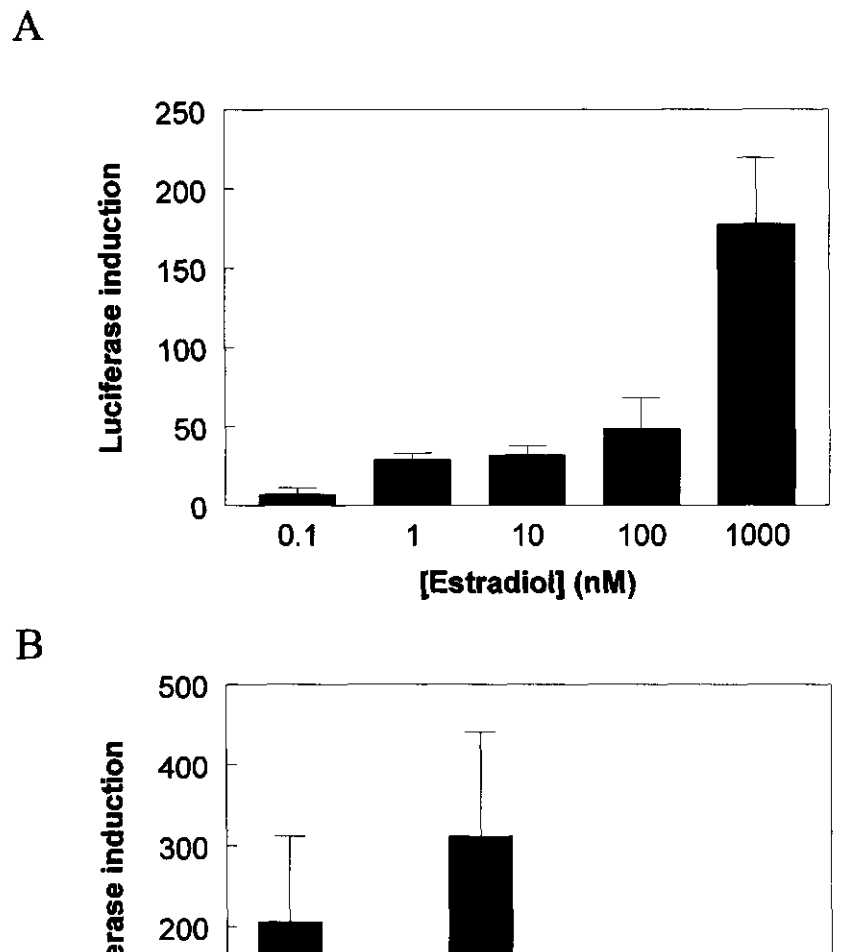


Figure 2: Luciferase activity (fold induction in light units/μg protein relative to vehicle exposed controls) in juvenile (35 days post fertilization) transgenic zebrafish exposed for 96 hours to (A) increasing doses of 17β-estradiol (E2) and (B) 1000 nM of the estrogenic compounds 17α-estradiol (E2-17A), estrone (E1), diethylstilbestrol (DES), ethinylestradiol (EE2) and o,p' DDT (DDT). Bars show mean luciferase induction in individual fry (n=3-4); error bars show standard error of the mean.

Ligand specificity and tissue distribution of testicular reporter gene induction

To gain further insight in the possible significance of the testicular estrogen receptors, we characterised the sensitivity of the transgenic testis to estradiol. A dose-response related luciferase induction to E2 was measured in testis of adults exposed to nominal concentrations of 0.1 to 1000 nM for 96 hours (Fig. 4a). During this short exposure period, 1 nM E2 already resulted in induced luciferase activity in the adult testis. To localize luciferase-producing cells in the testis, we incubated sectioned tissues of E2-exposed transgenic zebrafish with a polyclonal antibody against luciferase (Fig. 4b). Immunohistochemistry of the testis revealed positive staining in the Sertoli cells and early spermatogonia (germ cells), but not in the differentiated spermatocytes and spermatids (Fig. 4b-a,b). No staining was found in non-induced transgenic or wild type controls (Fig. 4b-c, 4b-e), as well as transgenic controls without luciferase antibody (Fig. 4b-d). Though little is known about the cellular localization of ER subtypes in fish testis, ER has been detected in the testis of goldfish (Tchoudakova *et al.*, 1999) and channel catfish (Xia *et al.*, 1999). Localization of luciferase-producing cells in the E2-induced transgenic testis is consistent with the ER distribution found in rodent testis, in which differential expression of ER-α and β has been found in various cell types, including Sertoli cells and spermatogonia (Van Pelt *et al.*, 1999, Saunders *et al.*, 1998). At the moment, the functional significance of highly responsive estrogen receptors in male reproductive tissues in unclear. However, luciferase induction in the spermatogonia suggests that estrogens may play a role in the maturation of germ cells. Induction of luciferase in the Sertoli cells may also be consistent with a role in testis size and sperm production.

Our results demonstrate that the testis may be the one of the most sensitive direct target organs for estrogenic pollutants. Although more detailed dose-response studies are necessary, our results with the transgenic zebrafish also suggest that the liver was two orders of magnitude less sensitive to exogenous estrogens than the gonads. Recent studies have demonstrated an estrogen receptor in cytosolic and nuclear fractions of the testis of the marine teleost Atlantic croaker (*Micropogonias undulatus*) which binds estrogens and xeno-estrogens with higher affinity than the hepatic ER (Loomis and Thomas, 1999). The occurrence of intersex gonads in wild populations of fish, which is associated with exposure to (xeno-)estrogens (Jobling *et al.*, 1998), also suggests the sensitivity of the gonads to environmental estrogens. We favour the hypothesis that estrogens can directly influence testicular functioning, and may even directly regulate the occurrence of intersex. Our future studies aim at testing this hypothesis, thereby testing the validity of the transgenic zebrafish model. Further studies will also be carried out to compare the usefulness of the transgenic zebrafish assay with another widely used biomarker for estrogenic effects in fish, namely the induction of the estrogen-regulated liver protein vitellogenin (Sumpter and Jobling, 1995).

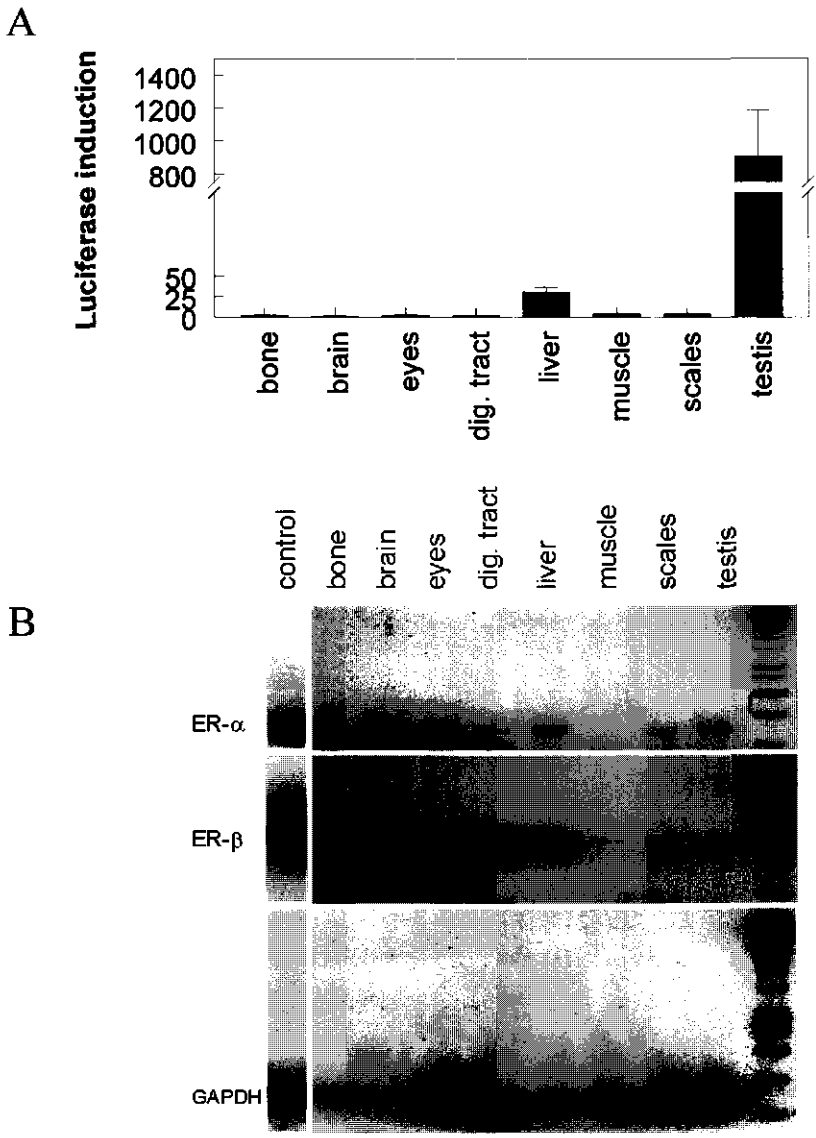
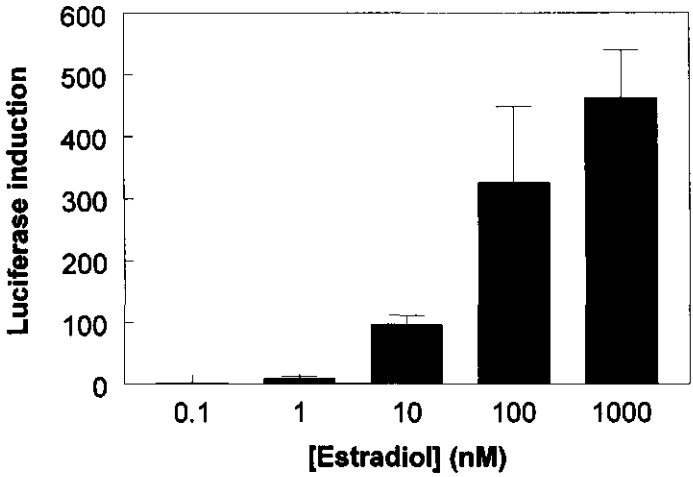


Figure 3: (A) Luciferase activity in tissues of adult male transgenic zebrafish exposed for 48 hours to 1000 nM E2 (fold induction in light units/µg protein relative to vehicle exposed controls) and (B) ***tissue-related expression of zebrafish estrogen receptor type ER-α and ER-β mRNA*** in non-exposed adult male zebrafish. "Control" lane shows PCR product from liver cDNA (10000 copies) amplified under the same conditions.

A



B

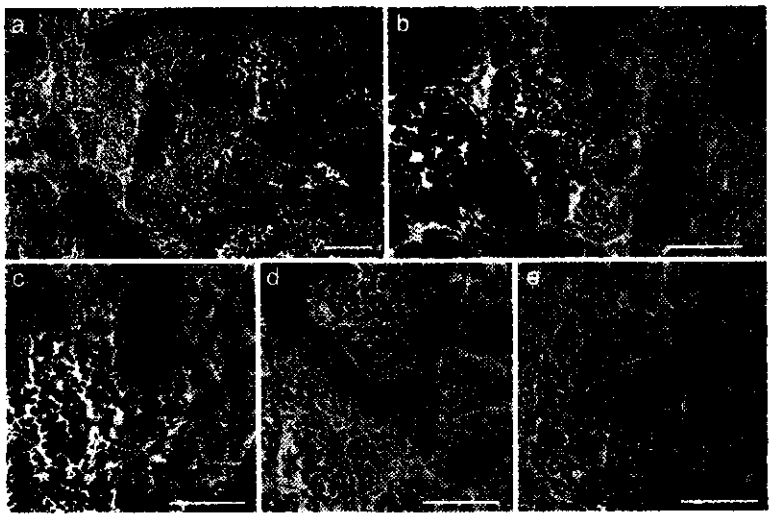


Figure 4: (A) Luciferase activity (fold induction in light units/μg protein relative to vehicle exposed controls) in testis of adult transgenic zebrafish exposed for 96 hours to increasing doses of E2. (B) Luciferase protein expression in testis of adult transgenic zebrafish exposed for 48 hours to 1,000 nM E2. Immunohistochemical staining of paraffin-embedded sections using polyclonal anti-luciferase antibody, showing (a, b) expression of luciferase localized in clusters of primary spermatogonia and Sertoli cells (brown stained cells). No background staining is found in: (c) non E2-induced transgenic testis; (d) negative controls without primary antibody; and (e) wild type non-transgenic testis (Bar = 50 μM).

In conclusion, we have developed a new *in vivo* model that has the potential to rapidly determine the estrogenic mode of action of chemicals during development and further throughout sexual differentiation in fish. In addition, we can determine whether these chemicals exert their effects directly on the testis or other tissues during adult stages. Thus the magnitude, tissue distribution and sensitive period of estrogenic effects can be determined *in situ.* As a complement to the wide variety of *in vitro* assays currently available to screen compounds for estrogenic activity (Zacharewski, 1997; Legler *et al.*, 1999), this transgenic model more realistically reflects the availability, kinetics, and metabolism of compounds *in vivo*. In comparison to long term reproduction tests, the transgenic zebrafish model may also offer a more rapid and specific means for screening substances with a suspected estrogenic mode of action. The transgenic zebrafish bioassay is a promising new tool in the field of environmental contaminant monitoring and risk assessment of new and existing chemicals.

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Chapter 6

Value of *in vivo* transgenic zebrafish and *in vitro* reporter gene assays for determination of estrogenic activity in environmental matrices

Submitted to *Environmental Science and Technology*

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Abstract

Functional *in vitro* and *in vivo* reporter gene assays have recently been developed to measure biological exposure to (xeno-)estrogens. The *in vitro* estrogen receptor (ER)-mediated chemical activated luciferase gene expression (ER-CALUX) assay uses T47D human breast cancer cells stably transfected with an ER-mediated luciferase gene construct. In the *in vivo* assay, transgenic zebrafish are used in which the same luciferase construct has been stably introduced. The transgenic zebrafish assay ensures that environmental chemistry and toxicokinetics of a substance *in vivo* are taken into account. Considerable differences were found in the relative potencies of the (xeno-)estrogens estradiol (E2), estrone, ethynylestradiol (EE2), o,p-DDT, nonylphenol (NP), bisphenol A (BPA) and di-(2-ethylhexyl)phthalate (DEHP) *in vitro* and *in vivo*. EE2 was the most potent of the (xeno-)estrogens tested and was 100 times more potent than E2 in the transgenic zebrafish assay whereas in the ER-CALUX assay, EE2 was only slightly (1.2 times) more potent than E2. Although the xeno-estrogens o,p'DDT, NP and BPA were full estrogen agonists in the ER-CALUX assay, only o,p'DDT demonstrated dose-related estrogenic activity *in vivo*. DEHP was only weakly estrogenic in both assays at concentrations above 1 μM. Estrogenic potency was determined in mixtures of (xeno-)estrogenic compounds in wastewater treatment plant (WTP) effluent as well as WTP effluent extracts and revealed levels of (xeno-)estrogens that exceed threshold values for negative effects in fish. Our results demonstrate that the transgenic zebrafish assay can provide a rapid and valuable tool to complement *in vitro* screening of estrogenic substances as well as indicating if substances may be estrogenic in fish.

Introduction

The presence of hermaphrodite fish has been increasingly reported in wild fish populations in rivers, estuaries and coastal waters (Lye *et al.*, 1997; Jobling *et al.*, 1998; Allen *et al.*, 1999; Vethaak *et al.*, 2000). These effects have been associated with exposure of fish in the aquatic environment to natural and xenobiotic chemicals with the same mode of action as the estrogen steroid hormones (the so-called "(xeno-)estrogens") (reviewed in Tyler *et al*, 1998). The focus on (xeno-)estrogens in the aquatic environment can be largely attributed to the finding that effluents from wastewater treatment plants (WTP) contain (xeno-)estrogenic chemicals at levels sufficient to invoke biological effects in fish in laboratory studies. Exposure of fish to WTP effluent has resulted in synthesis of the estrogen-inducible yolk precursor protein vitellogenin in male fish (Folmar *et al.*, 1996; Harries *et al.*, 1997, Rodgers-Gray, *et al.*, 2000) as well as changes in sexual differentiation of juvenile fish (Bulder *et al.*, 2000). The natural estrogens 17β-estradiol and estrone have been detected in WTP effluent at levels up to 80 ng/l (Desbrow *et al.*, 1998). These levels have been shown to stimulate vitellogenin synthesis in laboratory fish (Routledge *et al.*, 1998, Panter *et al.*, 1998). The synthetic estrogen 17α-ethynylestradiol (EE2) used in the contraceptive pill has also been detected in WTP effluents, at levels up to 7 ng/l (Desbrow *et al.*, 1998; Belfroid *et al.*, 1999a). Exposure of EE2 at concentrations of above 1 ng/l can stimulate synthesis of vitellogenin (Purdom *et al.*, 1994, Sheahan *et al.*, 1994) as well as retard growth and development of testes

in maturing male trout (Jobling *et al.*, 1996). Xeno-estrogens such as the alkylphenol surfactants have also been found in WTP effluent (reviewed in Nimrod and Benson, 1996). Nonylphenol, one of the microbial breakdown products of alkylphenol ethoxylates, has been detected in WTP effluent at levels that induce vitellogenin synthesis and decrease growth rate of testis (Jobling *et al*, 1996). Laboratory exposure to nonylphenol has also shown development of intersex conditions in male fish (Gray and Metcalfe, 1997).

Wastewater treatment plant effluent is a complex mixture of many (unknown) chemicals and routine chemical analysis of (xeno-)estrogens in effluent is hampered by high costs. Only known compounds can be analyzed and no account is taken of the biological effects of mixtures of chemicals. The use of bioassays to rapidly screen the exposure and demonstrate potential effects of (xeno-)estrogens can be an ideal means to determine the total estrogenic potency of mixtures of substances. Recently, extensive efforts to develop screening assays to detect exposure of (xeno-)estrogenic compounds on wildlife have been undertaken (Zacharewski, 1997; Ankley *et al*, 1998, Andersen *et al.*, 1999). In our laboratories, two new assays for estrogenic activity have been recently developed: the *in vitro* Estrogen Receptor-mediated Chemical Activated LUciferase gene eXpression (ER-CALUX) assay using human T47D breast cancer cells (Legler *et al.*, 1999), and the *in vivo* transgenic zebrafish assay (Legler *et al.*, 2000a). In both assays, an ER-mediated luciferase reporter gene construct containing 3 estrogen response elements (ERE) has been stably introduced and integrated in the genome of the T47D cells and transgenic zebrafish. In the ER-CALUX assay, exposure of cells to (xeno-)estrogens results in diffusion of chemicals through the cell membrane, binding to the endogenous ER, activation of the receptor, and consequently, binding of the ligand-receptor complex to EREs present in the promoter region of the luciferase gene (Figure 1). Luciferase protein is then induced, and is easily measured by lysing the cells, adding luciferin substrate, and measuring light photon production. In the transgenic zebrafish assay, luciferase is induced according to the same principle, but exposure to test substances takes place via the water phase. Therefore the environmental chemistry, bioavailability and toxicokinetics of the test substance *in vivo* determine ultimate exposure of target cells in the transgenic fish (Figure 1). Luciferase protein will only be induced in target cells in which the test substance is bioavailable, and active endogenous ER's and co-factors necessary for ER transcription are present. Therefore, in the transgenic zebrafish, tissue and life stage-specific effects of (xeno-)estrogens can be determined.

In this study, the ER-CALUX and transgenic zebrafish assays were applied to determine the *in vitro* and *in vivo* estrogenic potency of mixtures of (xeno-)estrogens in a domestic WTP effluent. For this purpose, individual (xeno-)estrogens that have been measured in WTP effluent were tested in both assays to determine the differences in relative potencies of these substances, and to determine if the mixture effects could be predicted by knowledge of the individual components of a mixture. The ability of the *in vitro* ER-CALUX assay to predict estrogenic effects *in vivo* is discussed.

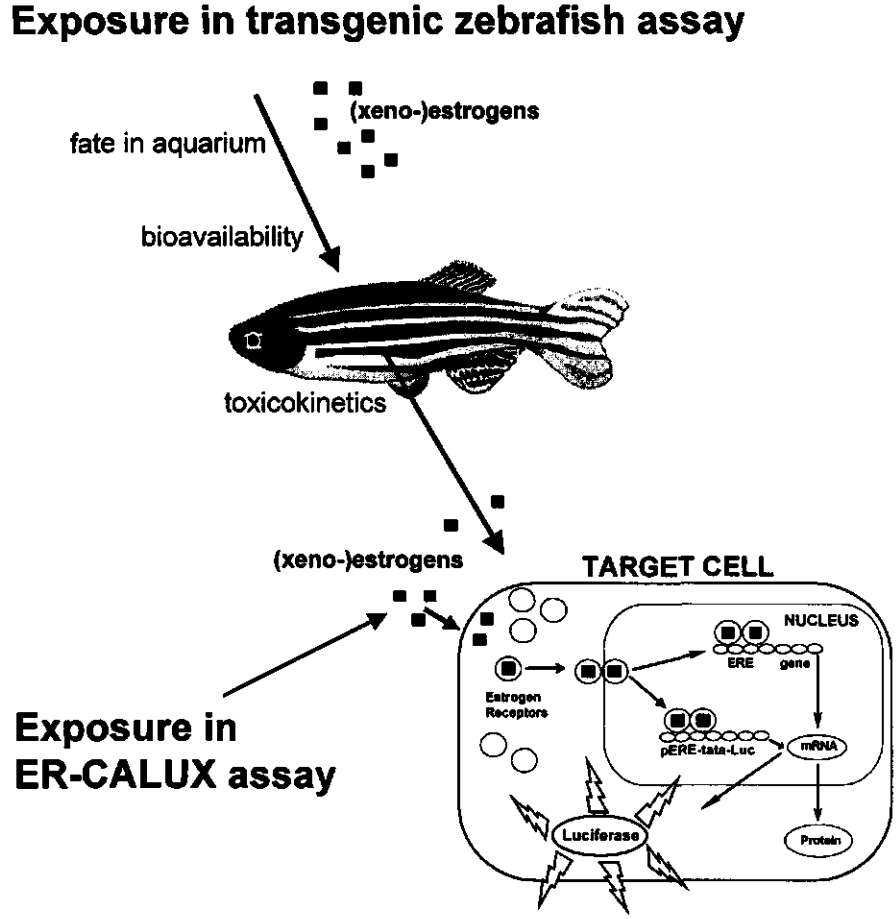

Figure 1: Principle of reporter gene (luciferase) induction in the in vivo transgenic zebrafish assay and the in vitro ER-CALUX assay in T47D cells. Differences in exposure routes in the two assays are illustrated. In the transgenic zebrafish assay, zebrafish are exposed to (xeno-)estrogens via the water phase. Bioavailability of (xeno-)estrogens depends on environmental chemistry and fate of the (xeno-)estrogen in the aquarium. In the fish, the compound may be metabolized, accumulated, eliminated and transported to target cells according to the toxicokinetics of the (xeno-)estrogen. In the ER-CALUX assay, cells are exposed directly to (xeno-)estrogens. Luciferase induction in target cells depends on the binding of xeno-estrogens to endogenous estrogen receptors which are then activated and bind to estrogen response elements (ERE) in the promoter regions of genes, finally resulting in production of luciferase. Luciferase will emit light photons when the substrate luciferin is added to cell homogenates.

Materials and Methods

Test substances

17β-estradiol (E2, 99%) and ethanol (100%, p.a.) were purchased from Sigma Chemicals Co. Estrone (E1, p.a.) was from Brunschwig. Ethinylestradiol (EE2, 98%) and Bisphenol A (BPA, 99%) were purchased from Aldrich. 4-n-nonylphenol (NP, 91%) and di-(2-ethylhexyl)phthalate (DEHP, 98% purity) were from TCI, Japan. o,p'-dichlorodiphenyl trichloroethane (o,p'DDT) was kindly provided by the Dutch State Institute for Quality Control of Agricultural Products (RIKILT-DLO). Nonylphenol ethoxylates (NP-4-E, average ethoxylate chain length of 4 units) and octylphenol ethoxylates (OP-8/9-E, average 8 to 9 ethoxylate units) were gifts from Shell. Dimethyl sulfoxide (DMSO, 99.9%, spectrophotometric grade) was purchased from Acros.

Synthetic WTP mix and WTP extracts

The levels of a number of known (xeno-)estrogens in the domestic effluent of a wastewater treatment plant (WTP) in a large city in the Netherlands were analyzed in a previous study (Belfroid *et al.*, 1999). Based on this information on concentrations of (xeno-)estrogens in the effluent, a synthetic mix of (xeno-)estrogens was made, consisting of three groups of compounds: 1) estrogens and bisphenol A; 2) nonylphenol and alkylphenol ethoxylates and 3) the phthalate DEHP. All compounds were dissolved in ethanol (p.a.). Group 1 consisted of 1.99 mg/l E1, 1.13 mg/l EE2 and 1.6 g/l BPA, dissolved in ethanol (p.a.). Group 2 consisted of 0.8 g/l NP, 3.71 g/l NP-4-ethoxylates (NP-4-E) and 0.2 g/l octylphenol-8/9-ethoxylates (OP-8/9-E). Group 3 consisted of 1.064 g/l DEHP. The three concentrated stocks were further diluted in ethanol in order to reach levels based on previous analysis of the actual effluent (Belfroid *et al.*, 1999a and 1999b).

In addition to the synthetic mix, extracts were made of the same WTP effluent as well as the receiving surface waters of the river at the WTP outfall, though at different sampling periods. Effluent samples (200 ml) were collected twice weekly in the period of September to November 1999, and frozen in glass bottles at –20°C. A 10 L sample WTP effluent-receiving waters was taken in April, 2000, stored at 4°C, and extracted within 3 days. Prior to extraction, effluent samples were thawed and mixed well and extracted using a previously described solid phase extraction method (Struijs *et al.*, 1998). Briefly, effluent and effluent-receiving water samples of 6 liters were mixed for 24 hours with macro-reticular resins (XAD). The XAD was then sieved and dried at room temperature for 12 hours. (Xeno-)estrogens were extracted by elution with acetone. The acetone fraction was evaporated under a gentle N2 gas flow at room temperature, and taken up in 60 µl DMSO. The DMSO fraction was further diluted for testing in the ER-CALUX and transgenic zebrafish assays.

ER-CALUX assay

The ER-CALUX assay procedure is described in detail elsewhere (Legler, *et al.*, 1999). Briefly, T47D.Luc cells stably transfected with pEREtata-Luc were plated in clear plastic 96 well plates (Nucleon, Denmark) at a density of 5000 cells in 0.1 ml DMEM-F12 without phenol red + 5% DCC-FBS per well. Following 24 hours incubation, medium was

renewed and the cells incubated for another 24 hours. The medium was then removed and the cells were dosed in triplicate by addition of the dosing medium containing the chemical or extract to be tested dissolved in ethanol or DMSO (max. 0.2%). Control wells, solvent control wells and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. Following 24 hours treatment, cells were lysed in 50 µl triton-lysis buffer, pH. 7.8 (containing 1% triton X-100, 25 mM glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT) for a minimum of 1 hour with gentle shaking at 4°C. A sample of 25 µl lysate was then transferred to a black 96 well plate (Costar) and 25 µl of luciferin solution (Luclite, Packard) was added per well. Luciferase activity was assayed in a scintillation counter (Top Count, Hewlett-Packard) for 0.1 minute per well.

Transgenic zebrafish assay

The recent development of the transgenic zebrafish is described elsewhere (Legler *et al.*, 2000a). For the assays, heterozygous transgenic juveniles of the F4 generation of the age of 4-5 weeks were used. Juvenile transgenic zebrafish undergoing gonad differentiation were used because this period was previously shown to be the most responsive to estrogens during development (Legler *et al.*, 2000a). Juvenile fish (n=5-6) were exposed for 96 hours in 150 ml acclimated tap water (26-27°C) in all-glass aquaria. The chemical or extracts to be tested was added to the water at volumes not exceeding 0.01% ethanol. Fish were fed once daily with live brine shrimp (*Artemia salinas*). Test medium was renewed for 50% daily. After termination of the exposure, fish were sacrificed in ice water, transferred to eppendorf vials, and immediately frozen at –80°C. To assay luciferase, eppendorf vials containing fish were transferred to ice, 500 µl of cold triton-lysis buffer added, and the fish homogenised using a micro-pestle (Eppendorf). Following centrifugation at 12.000 rpm for 15 minutes at 4°C, duplicate samples of 25 µl supernatant were assayed in a luminometer (LUMAC) with automatic injection of 100 µl luciferin substrate, pH 7.8 (containing 20 mM tricine, 1 mM $(MgCO_3)Mg(OH)_25H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.7 mM DTT, 270 µM coenzyme A, 470 µM luciferin and 530 µM ATP). Light units measured by the luminometer were normalized for protein content, which was measured according to Bradford, 1976.

Analysis of actual exposure concentrations

The hormones E2, E1 and EE2 as well as the xeno-estrogens NP and o,p'DDT were measured in test medium at the start (t=0) of an exposure experiment, prior to introducing the transgenic zebrafish, as well as at the end (t=96 hrs) of the experiment. Test medium samples were approx. 100 ml. NP and o,p'DDT levels were also analyzed in whole fish homogenates (n=5, total weight about 100 mg) at the start and termination of the experiment.

E2, E1 and EE2 analysis in water was carried out as described in Belfroid *et al.* (1999), with the exception that the quantification of the chromatograms was done with the internal deuterated standard of *d*4-17β-estradiol. For NP analysis in test medium, water was extracted according to the method described by De Voogt *et al* (1997). Analysis of NP in zebrafish following 96 hour exposure was done according to Zhao *et al.* (1999). Extracts were analysed by reversed-phase HPLC with fluorescence detection. For o,p'DDT analysis, test

medium samples from the were extracted three times with n-hexane. The extracts were dried over anhydrous Na_2SO_3 and analyzed by GC-ECD. Zebrafish were mixed with anhydrous Na_2SO_3 and then extracted three times with acetone:n-hexane (1:3 v:v). The extract was then filtered and analysed with GC-ECD. Extraction efficiency of o,p'DDT was 90-100%.

Data analysis

The EC50 values for both the ER-CALUX and transgenic zebrafish assay were calculated from dose-response curves using the curve-fitter of SlideWrite 4.0 (cumulative fit). The R of the fit of the curves was above 0.99 for the ER-CALUX assay, and above 0.9 for the transgenic zebrafish assay. EC50 values were calculated by determining the concentration at which 50% of the maximum luciferase activity was reached. For quantification of the estrogenic potency of an extract or component of the synthetic mix, the response of the extract was interpolated in the dose-response curve of the standard E2 curve. The extracts were diluted (for the ER-CALUX assay) or concentrated (for the transgenic zebrafish assay) to ensure that the responses could be interpolated within the linear portion of the curve. For the ER-CALUX, this was between the signal of 1 and 6 pM E2, and for the transgenic zebrafish assay, between 1 and 10 nM E2. The estrogenic potency of the extract is expressed as EEQ (estradiol equivalents) per volume of material. Estradiol equivalent factors (EEF) were determined as the ratio EC50 E2: EC50 test compound. EEQ and EEF values were calculated based on an average of 3 to 5 independent experiments.

Results and Discussion

Assay characteristics

The *in vitro* ER-CALUX assay using stably transfected T47D cells can detect estradiol (E2) at concentrations as low as 0.5 pM and is highly reproducible, with a coefficient of variation of about 5%. Maximal induction (about 100 fold relative to solvent controls) is reached at 30 pM E2. The *in vivo* transgenic zebrafish assay using 96-hour exposed juvenile transgenics has a detection limit of about 300 pM E2 and exhibits higher variation (CV=20-30%). Maximal fold induction of 1000-3000 fold relative to solvent controls is reached at 100 nM E2. The high biological variation is a reflection of individual fish differences in uptake and metabolism of substances, as well as differences in (gonad) developmental stage and sex. As the phenotypic sex of the transgenic zebrafish is not apparent at the age of fish used, both males and females are assayed together, thereby contributing to the variation in response. Previous studies have shown that male and female transgenics respond differently to E2 exposure (Legler *et al.*, 2000a). Interestingly, a decrease of luciferase induction by high concentrations of estrogens can be seen in both assays, particularly in the transgenic zebrafish assay (Figure 2). Down-regulation of ERs or other mechanisms of negative feedback to high dosages may cause this decrease.

Relative responses of the assays to estrogens

The ER-CALUX and transgenic zebrafish reporter gene assays demonstrate a dose-response-related increase in luciferase induction following exposure to estradiol, estrone (E1) and ethynylestradiol (EE2) (Figure 2). However, the sensitivities of the response of the two assays differ as well as the relative potencies of (xeno-)estrogens vary according to the assay used (Table 1). In the ER-CALUX assay, the EC50 of E2 and E1 were two to three orders of magnitude lower than in the transgenic zebrafish (Table 1). There may be several reasons for this difference in sensitivity. In the transgenic zebrafish assay, actual target cell exposure will likely be much lower than in the ER-CALUX assay, depending on the fate of the compound in the aquarium and its toxicokinetics in the fish. Chemical analysis of actual E2 and E1 levels in exposure water revealed that though actual concentrations were similar to nominal concentrations at the beginning of the experiment, very low amounts of these estrogens were present after 96 hours of exposure (Table 2) despite the fact that half of the exposure water was renewed daily. The disappearance of E2 and E1 from the water column is likely due to rapid uptake by the fish over the gills. *In vivo*, E2 and E1 can be extensively transformed to less potent metabolites by fish (Bone *et al.*, 1995). Therefore, in the transgenic zebrafish, higher concentrations of E2 or E1 are required to induce luciferase in target cells. In the ER-CALUX assay, the extent metabolism during the 24-hour exposure period is only slight. An additional reason for the difference in response to E2 and E1 can be found at the level of the ER. Fish ER's may have a lower affinity for E2 than mammalian ER's. Rainbow trout ER has been shown to require 10 times higher E2 concentration than the human ER for transactivation (Petit *et al.*, 1995; Le Drean *et al.*, 1995).

Interestingly, E1 induced luciferase in the transgenic zebrafish at similar concentrations as E2 (EEF=1, Table 1). In the ER-CALUX assay, however, E1 demonstrated an EEF of 0.23 (Table 1). These results indicate that E1 may be a more potent estrogen in fish than in mammals. While E1 binds with only 10% affinity to the rat ER relative to E2 (Blair *et al*, 2000), no difference was found in the ability of E1 and E2 to transactivate two rainbow trout ER isoforms of the trout ER gene (Pakdel *et al*, 2000). Accordingly, a recent study by Panter and colleagues demonstrated that E1 induced vitellogenin at concentrations similar to E2 in male fathead minnows (Panter *et al.*, 1998). Routledge and colleagues (1998) also showed that E1 was only slightly less potent than E2 in inducing vitellogenin in rainbow trout.

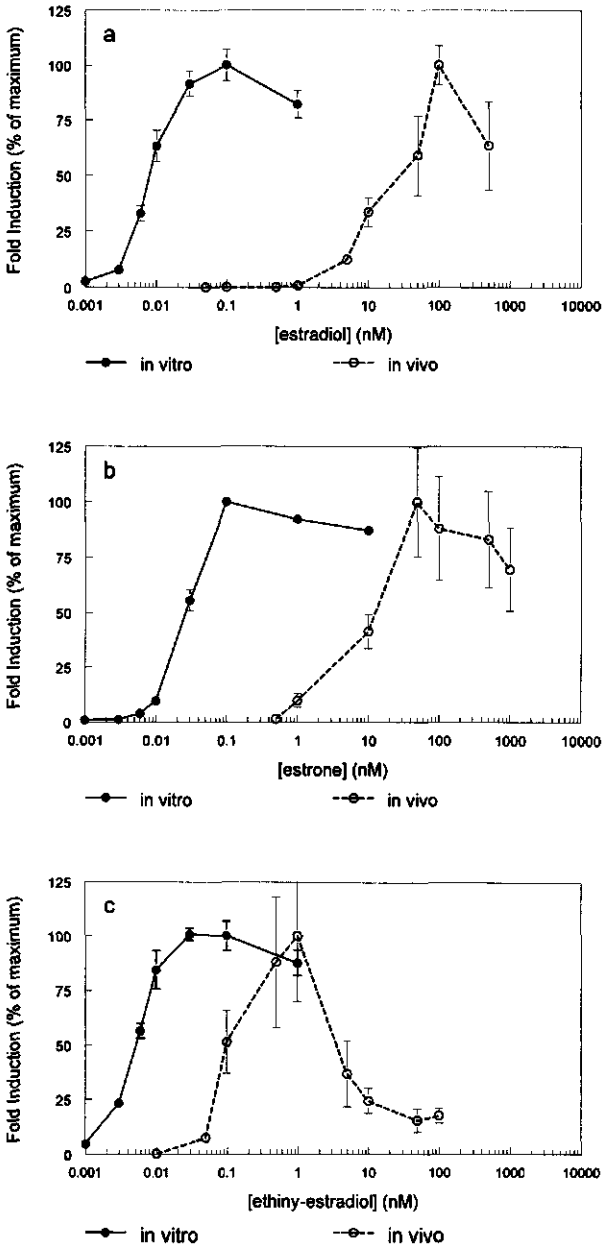

Figure 2: Estrogenic potency of (a) estradiol, (b) estrone, and (c) ethinyl-estradiol in in vitro ER-CALUX and in vivo transgenic zebrafish reporter gene assays. Data are representative of 3 independent assays. Values given are averages, error bars represent standard error of the mean (ER-CALUX: n=3; transgenic zebrafish: n=5-6).

In both assays, ethynylestradiol (EE2) was the most potent (xeno-)estrogen tested with only a 20-fold difference in EC50 between the ER-CALUX (0.005 nM) and transgenic zebrafish (0.1 nM) assays (Table 2, Figure 1c). Interestingly, in the transgenic fish, EE2 was 100 times more potent than E2 while in the ER-CALUX EE2 was 1.2 times more potent (Table 2). The high potency of EE2 in both assays can be explained partly by the high ER binding affinity of EE2. In rat uterus, EE2 exhibits over 2 times higher binding affinity to the ER than E2 (Blair *et al.*, 2000). In binding studies with channel catfish ER, EE2 was found to be five times more potent than E2 (Nimrod and Benson, 1997). Importantly, because EE2 is poorly metabolized by the liver and subject to intensive enterohepatic recycling (Guengerich, 1990), it is more resistant to metabolism *in vivo* than E2 and E1. Chemical analysis of the actual EE2 concentrations in the exposure water of the assay revealed that although EE2 was present at the nominal concentration at the beginning of the experiment, no EE2 was measured above the detection limit (20 ng/l) at the end of exposure (Table 2). This indicates that EE2 may be highly taken up by transgenic fish and is persistent *in vivo* where it exerts estrogenic activity to a higher degree than E2.

Table 1: Estrogenic potency of (xeno-)estrogens in *in vitro* ER-CALUX and *in vivo* transgenic zebrafish assays.

	ER-CALUX		Transgenic zebrafish	
compound	EC50 (nM)*	EEF**	EC50 (nM)*	EEF**
E2	0.006	1	10	1
EE2	0.005	1.2	0.1	100
E1	0.026	0.2	10	1
o,p'DDT	390	1.5E-05	450	0.02
NP	110	5.5E-05	0	0
BPA	770	7.8E-6	0	0
DEHP	0	0	0	0

*EC50 is nominal concentration at which 50% of maximal response is reached.
**EEF is ratio $EC50_{E2}$:$EC50_{compound}$.

Different responses of the assays to xeno-estrogens

The most dramatic difference in response between the ER-CALUX and transgenic zebrafish assay was found with exposure to xeno-estrogenic chemicals. Of the small panel of xeno-estrogens tested, only o,p'DDT induced luciferase activity in a full dose-dependent manner in both assays, and at similar nominal concentrations (Figure 3a, Table 1). Previous studies have also demonstrated the estrogenic activity of o,p'DDT in both fish and mammals (reviewed by Tyler *et al*, 1998). Chemical analysis of the exposure water and transgenic zebrafish (Table 2) revealed that actual o,p'DDT concentrations at the start and end of the experiment reflected nominal concentrations, and that o,p'DDT bioaccumulated in the fish. It should be noted that nominal and measured concentrations of o,p'DDT seemed to exceed the water solubility of this compound (about 250 nM). Because total extraction was carried out, DDT adsorption to particulates in the water was also measured.

In contrast to o,p'DDT, exposure to 4-nonylphenol only slightly induced luciferase in transgenic zebrafish (13 fold induction at 1000 nM or 1% of the response of 10 nM E2) while it was a full agonist in the ER-CALUX assay (Figure 3b). Nominal test concentrations above 1000 nM caused mortality of transgenic zebrafish. Chemical analysis of actual nonylphenol concentrations in exposure water revealed that only about 60% of the nominal concentration of NP was found at the start of the exposure period, prior to addition of the fish (Table 2), likely due to adsorption to glass walls. Following 96-hour exposure, only 7 µg/l NP of the nominal 220 ug/l NP was detected. Analysis of the fish following exposure revealed that NP was accumulated, though less than o,p'DDT. The low estrogenic potency of NP in the transgenic zebrafish is unexpected, considering the number of documented reports on the *in vivo* estrogenicity of nonylphenol (reviewed in Tyler *et al.*, 1998). NP has been shown to induce vitellogenesis and inhibition of testicular growth in male rainbow trout exposed under continous flow conditions for 3 weeks (Jobling *et al*, 1996) as well as induce feminized gonads in male Japanese medaka exposed during a full life cycle (3 months) (Gray and Metcalfe, 1997). It is possible that the 96-hour exposure duration of the transgenic zebrafish was too short to achieve an internal dose that could induce luciferase. NP has been shown to be readily metabolised in the liver of rainbow trout, with a half-life of 6 hours (Lewis and Lech, 1996). Recent studies in our lab have demonstrated that prolonged (3-week) exposure of adult male transgenic zebrafish to 100 µg/l NP resulted in elevated luciferase activity in the liver (Legler, unpublished results), suggesting that the short incubation period rather than the insensitivity of the transgenic fish caused the absence of a response to NP.

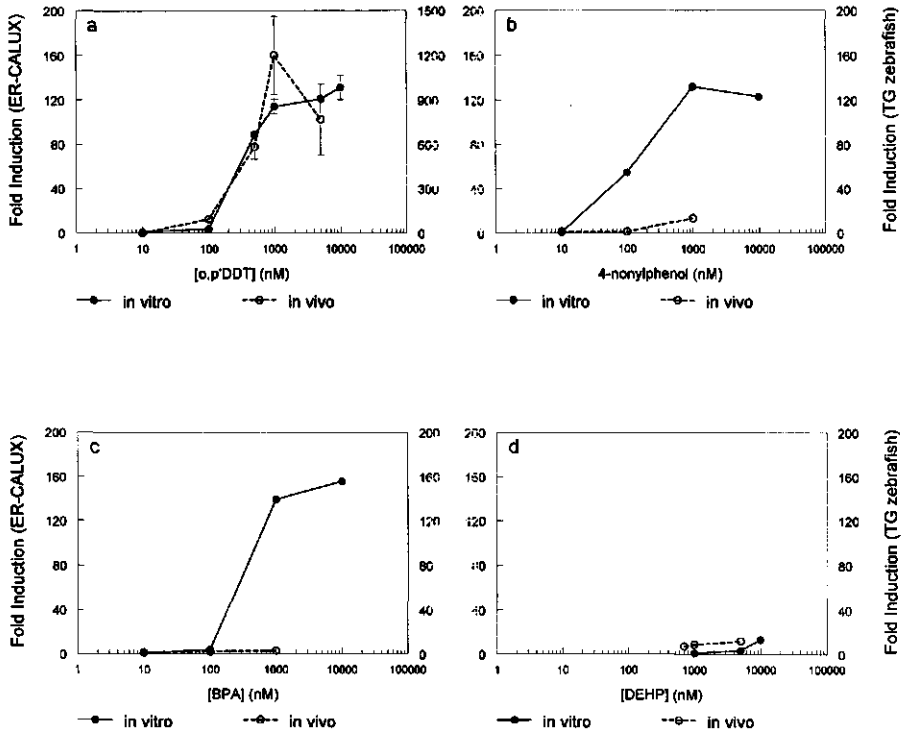

Figure 3: Estrogenic potency of xeno-estrogens (a) o,p'DDT, (b) 4-nonylphenol, (c) bisphenol A (BPA) and (d) DEHP in in vitro ER-CALUX and in vivo transgenic zebrafish reporter gene assays. Data are representative of 3 independent assays. Values given are averages, error bars represent standard error of the mean (ER-CALUX: n=3; transgenic zebrafish: n=5-6).

Bisphenol A provided an example of a xeno-estrogen that was not estrogenic at all in the transgenic zebrafish assay, though it induced dose-response mediated induction in the ER-CALUX assay (Figure 3c). Though chemical analyses of the actual concentrations of BPA in the water and fish were not performed, both short (96-hour) and prolonged 3-week exposure (Legler *et al.*, unpublished results) did not induce luciferase in the transgenic zebrafish. Though BPA has been previously shown to be estrogenic using mammalian *in vitro* and *in vivo* assays (Krishnan *et al.*, 1993; Steinmetz *et al.*, 1997), little has been reported regarding its estrogenic potency following waterborne exposure in fish. However, because a low bioaccumulation potential has been reported for BPA in the aquatic environment (Staples *et al.*, 1998), it is likely that target cell exposure in the transgenic zebrafish can not become high enough to induce estrogenic effects.

Table 2: Concentrations of (xeno-)estrogens determined in test water and fish taken during exposure experiments with transgenic zebrafish. Samples were taken at the start (T=0) and end (T=96hr) of the experiment.

	Nominal conc.		Measured concentrations			
	Water		*Water*		*Zebrafish*	
Comp.	nM	µg/l	(µg/l) T=0	T=96hr	(µg/kg) T=0	T=96 hr
E2	100	27	20.3	1	n.m.*	
EE2	1	0.3	0.4	<d.l**	n.m.*	
E1	100	27	30	0.2	n.m.*	
NP	1000	220	133	7	<d.l**	8940
o,p'DDT	1000	346	340	850	<d.l**	685000

*n.m.: not measured
**<d.l.: below detection limit

The phthalate DEHP demonstrated very weak estrogenic potency in both the ER-CALUX and the transgenic zebrafish assay (Figure 3d). In the ER-CALUX assay, 10 µM DEHP induced luciferase about 13 times above solvent controls (or about 13% of maximal E2 levels). In the transgenic zebrafish assay, 1000 and 5000 nM (nominal) DEHP induced luciferase about 10 times above solvent controls (or less than 1% luciferase induction relative to 10 nM E2). In both assays, no complete dose-response curves could be performed as concentrations above 10 µM resulted in cell toxicity in the ER-CALUX assay, and concentrations tested above 5 µM were toxic to the transgenic zebrafish. To our knowledge, specific estrogenic effects of DEHP in fish *in vivo* have not been demonstrated previously. The rapid metabolism of DEHP in rainbow trout has been demonstated (Barron *et al.*, 1987). In mammals, a recent study has shown the inability of DEHP to induce estrogenic activity (i.e. increase uterine wet weight) in rats *in vivo* (Zacharewski *et al.*, 1998). Earlier reports have demonstrated the potential of DEHP to induce reproductive organ toxicity in rats at high dosages only (Ganogolli, 1982; Davis *et al.*, 1994). These studies, taken together with our results, suggest that DEHP may not exert estrogenic effects at levels found in the environment.

Table 3: Estrogenic potency of (mixtures of) (xeno-)estrogens and wastewater treatment plant (WTP) extracts in the ER-CALUX (ERC) and transgenic zebrafish assay. Composition of compounds (comp.) and nominal concentrations tested were based on levels in WTP effluent. Estradiol equivalents (EEQs, pmol/l) are averages (±SEM) for 3-5 independent experiments.

	Nominal		ER-CALUX			Transgenic juveniles		
Comp. or	concentrations		Theoretical*		Measured	Theoretical*		Measured
Extract	ng/l	pM	ERC-EEF	ERC-EEQ	EEQs	FISH-EEF	FISH-EEQ	EEQs
1. Estrogens + BPA								
E1	5	18	0.2	4		1	18	
EE2	2.8	9	1.2	11		100	944	
BPA	4000	17520	8.E-06	0.1		0	0	
Sum EEQs				15	11 (0.5)		962	360 (110)
2. Alkylphenols								
NP	2000	9100	5.E-05	0.5		0	0	
NP-4-E	9300	24000	7.E-07[a]	0.0		n.m[b]	n.m	
OP-8/9-E	500	18000	0[a]	0.0		n.m	n.m	
Sum EEQs				0.5	0.4 (0.0)		0	10 (2)
3. Phthalate								
DEHP	2700	6910	0	0	0 (0.0)	0	0	8 (2)
Total mixture (containing 1-3)				16	10 (0.5)		962	570 (140)
WTP effluent extract[c]					12 (0.7)			189 (32)
WTP-receiving surface water extract[c]					9 (0.4)			237 (54)

*Theoretical estradiol equivalents (EEQ) were calculated by multiplying concentration (pM) by estradiol equivalency factor (EEF= ratio $EC50_{E2}$:$EC50_{compound}$).
[a] from Legler *et al.*, 2000c [b] not measured [c]concentrations of (xeno-)estrogens not measured

Environmentally realistic mixtures of (xeno-)estrogens

The estrogenic potency of a synthetic mix composed of (xeno-)estrogens at concentrations found in a domestic WTP effluent was tested, as well as an extracts of total effluent and receiving surface waters (Table 3). This particular WTP effluent was chosen as a model effluent for estrogenic effects because wild male bream sampled from the river receiving this WTP effluent showed elevated vitellogenin levels (Legler *et al.*, 2000c) as well as a high incidence of intersex gonads (Vethaak *et al.*, 2000). The synthetic mix was composed of three sub-groups: estrogens (E1, EE2) and bisphenol A; alkyphenols (NP and NP/OP ethoxylates); and the phthalate DEHP. The presence of free E2 itself was not detected in this WTP sample. The total theoretical estrogenic potency (EEQ) of this mix was calculated by summing the products of the EEF of the (xeno-)estrogens in the synthetic mix and their molar concentration (Table 3). In the ER-CALUX assay, the theoretical EEQs in the hormone and BPA group (15 pM) correlated well with the measured EEQs (11 pM), as did the alkylphenol group (theoretical EEQ 0.5 pM vs measured EEQ 0.4 pM) (Table 3). DEHP did not induce luciferase activity in the ER-CALUX assay. The measured EEQ (10 pM) in the total mix was in the same order of magnitude as the theoretical EEQ (16 pM) based on the EEF approach. These results on the total *in vitro* estrogenic potency of the mixture indicate that the individual estrogenic compounds behaved in an additive manner.

Higher estrogenic activity was found when testing the synthetic mix in the transgenic zebrafish assay than in the ER-CALUX assay (Table 3). The major reason for the relatively high estrogenic activity in the zebrafish is the probably the presence of EE2 in the mix, which is 100 times more potent than E2 and E1 in the transgenic fish (Table 1). The group composed of E1, EE2 and BPA contributed most to the total estrogenic activity measured (Table 3). Both the alkylphenol group and DEHP were slightly estrogenic in the transgenic fish assay (Table 3). Comparison of the total theoretical estrogenic activity of the synthetic mixture (960 pM EEQ) based on the zebrafish-EEFs of the individual compounds resulted in a value comparable to the actual measured activity (570±140 pM EEQ).

In addition to the synthetic mix, extracts were made from the actual effluent from the same WTP, as well as the WTP effluent-receiving surface waters. In the ER-CALUX assay, little difference was found in estrogenic activity between the whole effluent extract and the synthetic mix (12 and 10 pmol EEQ/l, respectively). Extracts of the WTP receiving surface water showed elevated EEQs in the ER-CALUX (9 pM) suggesting a minimal effect of dilution by surface waters. When tested in the transgenic zebrafish assay, both the whole effluent extract and the receiving waters extract showed about the same estrogenic activity (189 and 237 pM EEQ, respectively, Table 3), also suggesting that dilution by river water did not affect the levels of potent (xeno-)estrogens. However, in the transgenic zebrafish, the estrogenic activity in the whole effluent extract was about two to three times lower than estrogenic activity in the synthetic mix. Because this effluent was sampled at a different time period, it is possible that levels of potent (xeno-)estrogens in the fish, such as EE2, were different from those in the synthetic mix. Unfortunately, estrogen concentrations in the total effluent extracts were not measured, though temporal fluctuations in estrogen content in effluents have been shown previously (Rodgers-Gray *et al*, 2000, Belfroid *et al.*, 2000). The

in vivo estrogenic activity found in the WTP effluent is at a level in which negative effects on fish reproduction may be expected by exposure to this WTP effluent. EEQ values in the WTP effluent extracts exceed the threshold values of E2 and EE2 required to induce vitellogenin induction (Routledge *et al.*, 1998; Sheahan *et al.*, 1994), reduce testicular growth (Jobling *et al.*, 1996) as well as inhibit egg production (Kramer *et al.*, 1998) and development (Kime and Nash, 1999).

Use of in vitro and in vivo reporter gene assays for assessing estrogenic activity

Both the *in vitro* ER-CALUX assay and the *in vivo* transgenic zebrafish assay have a number of characteristics that make them useful screening tools. The ER-CALUX assay is extremely sensitive, rapid and can be used to screen many chemicals in a high-throughput manner. However, an *in vitro* assay can not predict the uptake and toxicokinetics of a substance *in vivo*. For this reason, the *in vivo* transgenic zebrafish assay forms an excellent complement to the ER-CALUX assay. The assay is rapid (96 hours), easy to perform, and the measured endpoint (luciferase activity) is simple and cost effective when compared to long-term exposure studies in which reproductive and histological parameters are analysed. The value of the transgenic zebrafish assay is that it can predict if estrogenic activity measured with an *in vitro* assay actually may affect fish during a critical life stage, e.g. the stage of gonad differentiation. When adult transgenic fish are used, it is possible to determine the tissue specific effects of (xeno-)estrogens (Legler *et al.*, 2000a).

In this study, the transgenic zebrafish assay demonstrated that less readily metabolized (xeno-)estrogens such as EE2 and o,p'DDT were more potent *in vivo* than would be expected based on their *in vitro* estrogenic activity. Xeno-estrogenic chemicals which are metabolized *in vivo* such as NP and BPA, were estrogenic in the mammalian cell *in vitro* but did not induce reporter gene expression *in vivo*. These results indicate that the ER-CALUX assay may predict the potency of a xeno-estrogen once it has reached the target cell, but it can not predict the bioavailability of the substance to the cell. However, caution should be exercised when assessing the apparent lack of estrogenic effects of some xeno-estrogens in the transgenic fish. Estrogenic activity *in vivo* depends on a number of factors including exposure duration and exposure route. More research is necessary to determine if the *in vivo* transgenic zebrafish assay may prove more responsive to xeno-estrogens by testing prolonged exposure duration under continuous flow conditions. In addition, some differences in response between the two assays may be explained by differences in mammalian and fish ER activation. Comparing the transgenic zebrafish assay with an *in vitro* reporter gene assay using zebrafish ERs would be more appropriate to exclude these differences.

In testing complex mixtures, the exquisite sensitivity of the ER-CALUX assay system makes it possible to detect estrogenic activity in extracts of small samples. The ER-CALUX assay is a suitable choice for screening complex environmental samples for unexpected estrogenic activity that could be further identified by chemical analysis, as well as further tested in the transgenic zebrafish assay to determine *in vivo* estrogenic potency. Knowledge of the chemical composition of (xeno-)estrogens in an environmental mixture, and the relative potencies (EEFs) of the identified (xeno-)estrogens in the transgenic zebrafish assay, can be

valuable to predict the possible effects of the mixture in fish without the need for long-term studies with many experimental animals. Ultimately, it is essential to determine what level of exposure to (xeno-)estrogens will result in reproductive problems in aquatic organisms that may eventually affect the survival of the population. Ideally, biological validation of the ER-CALUX and transgenic zebrafish assays will reveal critical levels of reporter gene induction which correspond with estrogenic effects on gonad differentiation and reproduction in the zebrafish.

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Chapter 7

General Discussion

In recent years, both scientific and public concern about the possible threat of estrogenic compounds in the environment which may impact the reproduction of humans and wildlife has increased. Many substances have been demonstrated to possess estrogenic potency using *in vitro* test systems, and these compounds have been identified in the environment using chemical analysis. However, up until now, it has been difficult to rapidly estimate the total biologically active estrogenic potency in environmental mixtures, as well as predict the exposure of and possible effects on organisms. The research presented in this thesis describes the development and application of two new bioassays to determine the biologically active exposure of natural and xenobiotic estrogens (xeno-estrogens) in the environment and predict the estrogenic effects of such exposure on fish. These new bioassays may help to determine if organisms are exposed to levels of (xeno-)estrogens which may impact their reproduction or endocrine functioning.

Summary of results

In vitro ER-CALUX reporter gene assay

The estrogen receptor (ER)-mediated chemical activated luciferase reporter gene expression (ER-CALUX) assay was developed by stable transfection of an ER-regulated luciferase reporter gene in the T47D breast cancer cell line (Chapter 2). The ER-CALUX is the most sensitive and highly responsive stably transfected vertebrate cell reporter gene assay for estrogens reported to date. Exposure (24-hour) to estradiol (E2) demonstrated a detection limit of 0.5 pM, an EC50 of 6 pM and a maximum induction of 100-fold at 30 pM relative to solvent controls. The presence of anti-estrogenic activity can be determined by co-incubation with E2, and was demonstrated for ICI 182,780, TCDD and tamoxifen. A variety of xeno-estrogenic compounds were shown to be estrogenic in the ER-CALUX assay, including pesticides, alkylphenols, phtalates and phytoestrogens, though with a potency 10,000 to 100,000 times less than E2 (Chapter 2 and 3). Interactive effects of the mixtures of xeno-estrogens were tested, and in general, additive effects were found (Chapter 2).

The response of the ER-CALUX assay was compared with the widely used recombinant yeast screen (Routledge and Sumpter, 1996) for determining estrogenic potency. The ER-CALUX assay is 10 to 100 times more sensitive to E2, nonylphenol and o,p'DDT. Unlike the ER-CALUX, yeast cells were unable to demonstrate the anti-estrogenic effects of ICI 182,780 and tamoxifen, as these compounds were agonistic in the yeast (Chapter 3). Because of these differences, the ER-CALUX assay may be more suitable for screening environmental samples in which the amount of material available for extraction is limited, or levels of (xeno-)estrogens are very low.

The inclusion of an *in vitro* biotransformation step in the ER-CALUX assay may assist in predicting the *in vivo* activity of (xeno-)estrogens. Pre-incubation of E2 and methoxychlor with liver microsomes obtained from PCB-treated rats demonstrated that metabolites of methoxychlor were more estrogenic than the parent compound (Chapter 3).

Given the exquisite sensitivity of the ER-CALUX assay, it can be an ideal screening tool for determining estrogenic activity in complex mixtures from various environmental matrices. Extracts of sediments were tested in the ER-CALUX assay for estrogenic potency. A number of sediment extracts obtained from industrialized areas such as the Port of Rotterdam showed high estrogenic potency (up to 40 pmol estradiol equivalents per gram sediment) (Chapter 3).

A simple method to determine if estrogen metabolites present in human urine and fish bile can be reactivated into active estrogens was developed (Chapter 4). Estrogen glucuronides in urine obtained from human males and females were deconjugated by enzymatic hydrolysis following incubation with ß-glucuronidase or live E. coli cells, indicating that bacterial hydrolysis as is present in wastewater treatment plants may form a secundary source of estrogenic substances in the environment. Also, glucuronides in bile samples from male fish showed estrogenic activity following deconjugation. These conjugated metabolites could also form a secondary source of estrogen exposure in fish due to enterohepatic recycling. High estrogenic activity of deconjugated bile metabolites from male bream correlated with elevated estrogenic potency in water extracts as well as vitellogenin

induction in blood of the same fish. Estrogenic potency of (deconjugated) bile and urine metabolites could form a useful biomarker for internal dose of (xeno-)estrogens in males (Chapter 4).

In vivo reporter gene assay using transgenic zebrafish

Transgenic zebrafish were developed by stably introducing an estrogen binding sequence linked to a TATA box and luciferase reporter gene into the genome. This is the first report of an *in vivo* reporter gene assay to assess exposure to estrogen modulating substances (Chapter 5). Exposure of the transgenic zebrafish to E2 during juvenile stages revealed the period of gonad differentiation to be the most responsive early life stage. In this juvenile period, E2 induced luciferase activity in a dose-dependent manner. Also the natural estrogens estrone and 17α-estradiol, as well as the synthetic estrogens diethystilbestrol and ethinylestradiol, and the organochlorine pesticide o,p'DDT demonstrated estrogenic activity *in vivo* in this test system. In adult male transgenic zebrafish, short-term exposure to E2 revealed a number of tissues such as liver, bone, muscle and scales to be a potential target organ for E2. However, the testis was the most sensitive and responsive target tissue to estrogens. Exposure to E2 resulted in dose-response related luciferase induction in the testis. Immunohistochemistry revealed the Sertoli and germ cells to be the main target cells of E2 in the zebrafish testis. ER α and β mRNA was detected already in young embryos (24 hours post fertilization) and both subtypes were highly expressed during the period of gonad differentiation. In adult male transgenics, tissues with the highest luciferase activity (testis and liver) expressed both ER α and β mRNA (Chapter 5).

Exposure of ER-CALUX cells is very direct without interaction with environmental factors influencing bioavailability and accumulation and without the toxicokinetics of an intact organism. In the transgenic fish assay, however, these factors do play a role, resulting in concentrations target cells that are sometimes difficult to predict using an *in vitro* system. Both assays exhibited high responsiveness to natural and synthetic estrogens such as E2, E1 and EE2, though differences in their relative potencies to E2 were found. EE2 was 100 times more potent than E2 in the transgenic fish, whereas it was only slightly (1.2 times) more potent than E2 in the ER-CALUX assay. The highly accumulating xeno-estrogen o,p'DDT was estrogenic in both assays, with similar potencies relative to E2. On the other hand, xeno-estrogenic chemicals with which are readily metabolized *in vivo* such as NP and BPA were estrogenic *in vitro* but did not induce reporter gene expression *in vivo* in short-term exposures (Chapter 6).

Estrogenic activity was detected in mixtures reflecting concentrations of (xeno-)estroens found in a domestic wastewater treatment plant effluent (WTP) as well as extracts from WTP effluent, using both the ER-CALUX and transgenic zebrafish assay (Chapter 6). Estrogenic potency in WTP effluent was higher in the transgenic zebrafish assay than in the ER-CALUX assay. This may have been due to the presence of individual estrogens such as EE2 which are more potent estrogens in the fish than in the *in vitro* system.

Perspectives for the use of in vitro and in vivo reporter gene assays in environmental toxicological research

The ER-CALUX and transgenic zebrafish assay are very promising complementary *in vitro* and *in vivo* biomarkers of exposure to and potential effects of substances with an estrogenic mode of action. The ER-CALUX assay is rapid and simple, and detection in microtiter volumes is possible, allowing for qualitative and quantitative assessment of luciferase induction in a high throughput setup. The assay can be used as a biomarker of internal exposure to (xeno-)estrogens as demonstrated for male fish bile samples. The ER-CALUX assay is a suitable choice for screening complex environmental samples for unexpected estrogenic activity that could be further identified by chemical analysis. This *in vitro* assay has proven to be very effective in detecting estrogenic activity in complex environmental mixtures, such as extracts of sediments (Chapter 3), water and biota (Chapter 4), and effluents (Chapter 6). For these reasons, the ER-CALUX assay is being implemented in various field surveys in The Netherlands to determine estrogenic activity in a wide variety of environmental matrices from various freshwater and marine locations (Murk *et al.*, 2000, Vethaak *et al.*, 2000). It is important to note, however, that when using extracts of environmental matrices, more chemicals will be extracted from an environmental matrix than may be realistically bioavailable to an aquatic organism, or there may be loss of compounds during the extraction procedure. Therefore, extraction methods used require further chemical and biological validation to identify which substances are responsible for estrogenic activity and to determine if these substances are bioavailable in aquatic systems.

Despite the many advantages of using *in vitro* assays to determine biological potency of (mixtures of) substances, one of the main disadvantages is their simplification of the *in vivo* situation. The *in vitro* ER-CALUX assay cannot completely reflect complex *in vivo* events, such as bioavailability and toxicokinetics, of a compound. The ER-CALUX assay may predict the potency of a (xeno-)estrogen once it has reached the target cell, but it can not predict the final bioavailability of the substance to the cell. Cross-talk with other mechanisms not directly related to the interaction of substances with the ER signal transduction pathway are not included in the ER-CALUX assay. The breast cancer cell line used in the ER-CALUX assay expresses both ERα and ERβ subtypes, which may be problematic for the prediction of tissue-specific effects. Tissues such as prostate and ovary have been found to contain prominent ERβ expression (Kuiper *et al.*, 1997). Differences in the binding affinities of compounds, such as phytoestrogens, as well as in the transcriptional activity for both ER subtypes have been demonstrated (Kuiper *et al.*, 1998). In addition, the possibility of extrapolating the estrogenic potency predicted in the ER-CALUX assay using human cells to other species, such as fish, birds and reptiles, requires further study. For example, differential binding affinities of polychlorinated biphenyls using human, rainbow trout and reptilian ERs have been recently demonstrated (Matthews and Zacharewski, 2000). Development of reporter gene assays using ERs in estrogen-sensitive cell lines from various species may be needed to better predict species-specific estrogenic effects in a particular species.

The *in vivo* reporter gene assay using transgenic zebrafish addresses many of the disadvantages associated with *in vitro* assays. In addition to being rapid and easy to perform, reporter gene induction in this assay is a reflection of the bioavailability and toxicokinetics of a substance *in vivo*. This assay provides a unique means to determine the effects of (xeno-)estrogens on target tissues, as well as critical life stages for estrogen exposure in the development of the zebrafish. Even with short-term exposure of juvenile transgenic zebrafish, considerable differences were found in relative estrogenic potency of compounds in the zebrafish *in vivo* than would be predicted according to *in vitro* estrogenic activity. In particular, substances that were resistant to metabolism *in vivo*, such as ethinynlestradiol, or highly accumulating, such as o,p'DDT, were more estrogenic in the zebrafish than would be predicted based on their *in vitro* estrogenic activity. This type of information increases insight in mechanisms underlying differences between *in vitro* and *in vivo* potencies needed to determine the potential risk of complex mixtures of chemicals to fish. If the principal differences between *in vitro* and *in vivo* estrogenic potencies are known for a well studied matrix, it may be possible to rely more on the fast, cheap and animal-friendly *in vitro* assay for large scale monitoring for this type of matrix. One example of a such as matrix are domestic wastewater treatment plant effluents in which natural and synthetic estrogens are the main source of estrogenic activity and their relative potency in the transgenic zebrafish is known. For environmental matrices in which the chemical composition is unknown or estrogenic potency *in vitro* is higher than expected, more research should be conducted on determining the estrogenic potency of individual compounds in the transgenic zebrafish.

A number of aspects require further study to realize the full potential of the transgenic zebrafish assay. As of yet, only a small panel of xeno-estrogens have been tested in the zebrafish. As some xeno-estrogens did not induce reporter gene activity under short-term static renewal regimes (Chapter 6), use of longer exposure periods under continous flow conditions may be useful. Parental transfer of (xeno-)estrogens could be studied by determining luciferase induction in offspring. The tissue-specific anti-estrogenic working of compounds could be determined by co-administration of estrogen with suspected anti-estrogens, or investigation in adult female transgenics, where luciferase levels by endogenous estrogens are elevated. In addition, a detailed comparison of the transgenic zebrafish assay with another widely used biomarker for estrogen exposure, namely vitellogenin induction, would shed light on the advantages and disadvantages of both test systems.

Possible impact of current levels of (xeno-)estrogens on fish

The final challenge for an environmental toxicologist is to determine whether the exposure of aquatic organisms to (xeno-)estrogens results in reproductive problems which affect the survival of wild populations. The presence of highly responsive ERs in the testis of adult transgenic zebrafish as well as in juveniles undergoing sexual differentiation (Chapter 5) suggests the sensitivity of the gonads to realistic estrogen exposure. Studies are ongoing to determine if reporter gene activity in adult males and sexually differentiating juvenile transgenic zebrafish can predict reproductive and gonad morphological effects. Preliminary studies have demonstrated that exposure to estradiol during juvenile life stages in which

luciferase reporter gene induction is highly elevated results in reduced fertilization success of adult male fish (Legler *et al.*, manuscript in preparation).

In this study, elevated *in vitro* and *in vivo* estrogenic activity was found in extracts from domestic effluent of a wastewater treatment plant in The Netherlands, as well as in the effluent-receiving surface waters (Chapter 6). Male fish sampled from the river receiving this effluent demonstrated both elevated biliary estradiol equivalents (EEQ) and plasma vitellogenin levels compared to reference sites (Chapter 4). In the transgenic zebrafish, approximately 200 pmol EEQ/l was measured following 96-hour exposure to these extracts. This level of EEQ far exceeds levels reported in the literature necessary to induce vitellogenin induction (Sheahan *et al.*, 1994, Routledge *et al.*, 1998) and inhibit testicular growth (Jobling *et al.* 1996, Panter *et al.* 1998) in other fish species. This indicates that fish at this particular location may be exposed to (xeno-)estrogens at levels that may impact their reproductive success. Accordingly, wild populations of bream sampled from the river receiving this WTP effluent showed high numbers of male fish with hermaphroditic gonads compared to reference locations (Vethaak *et al.*, 2000). Exposure of F0 and F1 generations of zebrafish to this effluent in partial life cycle studies also demonstrated adverse effects on sexual differentiation of juvenile zebrafish (Bulder *et al*, 2000).

Elevated estrogenic activity was found not only in water and effluent sample extracts using the ER-CALUX assay, but also in extracts of sediments from various locations in the Netherlands (Chapter 3). This demonstrates that (xeno-)estrogenic substances may also accumulate in sediments in the aquatic environment. The lipophilic pesticide o,p'DDT, which would be found more in sediment than the water phase in the aquatic environment, was the most potent xeno-estrogen of the compounds tested in the transgenic zebrafish assay so far. Therefore, more attention should be given to the estrogenic effects of hydrophobic compounds that may be available to aquatic organisms via food and through contact with particulate matter and sediment.

Concluding Remarks

This study resulted in the development of two important new biological tools to identify estrogenic compounds. These tools are very valuable in determining if substances in the environment are present at levels that may interfere with reproduction or endocrine functioning in organisms. The *in vitro* ER-CALUX assay with T47D cells is the most sensitive and responsive stably transfected reporter gene assay reported to date and has already proven indispensable in rapidly and cost-effectively determining total biological potency of estrogenic chemicals in complex environmental mixtures. The *in vivo* transgenic zebrafish assay is the ideal complement to the ER-CALUX assay because environmental chemistry and toxicokinetics of compounds are taken into account. This assay is important for predicting the estrogenic effects of chemicals on fish. The surprising result that the male testis, and not the liver, is the most sensitive and responsive target tissue to estrogens underlines the vulnerability of the male reproductive organs and provides a mechanistic basis for the worldwide observance of male gonad abnormalities in wild populations of fish. In addition to identifying sensitive target tissues, the transgenic zebrafish assay has been pivotal in demonstrating the period of gonad development in juvenile life stages to be the most responsive to exogenous estrogen treatment.

The possible applications of the ER-CALUX and transgenic zebrafish assay in the fields of mechanistic and environmental toxicology are numerous. For example, in environmental toxicology, the ER-CALUX assay can be a useful biomarker for internal dose of (xeno-)estrogens by determination of the estrogenic potency of (deconjugated) bile and urine metabolites of males. For mechanistic research, comparison of *in vitro* and *in vivo* bioassay responses can shed light on bioavailability, toxicokinetices and tissue-specific effects of (xeno-)estrogens. Of course, the route of exposure of (xeno-)estrogens and concentrations used in the transgenic zebrafish assay should be realistic. Injection of (xeno-)estrogens, as opposed to exposure via the water or particulate (food) phase, is not a realistic exposure route for fish and will overestimate the bioavailability of chemicals *in vivo*.

Unfortunately few well-validated *in vitro* test systems exist, and for this reason, the use of *in vivo* test systems has been widely promoted by regulatory agencies for endocrine disruption research. The result is that many experimental animals are sacrificed for what are generally labour intensive and expensive experiments. The use of the ER-CALUX assay and the transgenic zebrafish assay can drastically fine-tune and further direct complex *in vivo* studies. Pre-screening with the ER-CALUX assay will determine if a substance is a potential estrogen. Further testing in the transgenic zebrafish assay at concentrations not exceeding 5 μM (due to possible toxicity and exceeding water solubility levels) will determine if the substance is also estrogenic in fish. Determination of estrogenic effects of a substances in long-term reproductive studies would only be warranted if the estradiol equivalency factor (EEF) of the substance in the transgenic fish is greater than 10^{-6}. Ideally, future studies to biologically validate the transgenic zebrafish assay will determine critical levels of reporter gene induction in the zebrafish which correspond with effects on gonad differentiation and reproduction, thereby further reducing the need for chronic studies with large numbers of experimental animals.

In response to the question "can estrogenic effects be expected in aquatic organisms exposed to (xeno-)estrogenic compounds in the Dutch environment?", the answer is yes. Though probably not widespread, there are some localized areas of concern. In this study we showed that domestic wastewater treatment plant effluent is estrogenic in both the ER-CALUX and transgenic zebrafish assay. Natural and synthetic hormones contributed most to this estrogenic activity, in particular the contraceptive pill component ethynylestradiol (EE2). Importantly, EE2 was one hundred times more potent than the natural hormone estradiol in the transgenic zebrafish, demonstrating that only small amounts of this compound in the environment can have dramatic effects on fish. EE2 is definitely a priority estrogenic contaminant in the environment and measures should be taken to limit its release in domestic sewage effluent. In addition, another source of estrogenic activity in the environment that may be underestimated is sediment. Persistent compounds that likely enter the environment via industrial effluents or runoff from pesticide use are accumulated in sediments. Extracts from contaminated sediments, such as from Rotterdam Harbour, showed elevated estrogenic activity in the ER-CALUX assay. Aquatic organisms and their eggs can be exposed to sediment-associated (xeno-)estrogens through contact or ingestion of sediments. In this study, the organochlorine pesticide o,p'DDT which is mainly found accumulated in sediments and not in the water phase, was the most potent xeno-estrogenic chemical in the transgenic fish. More attention in terms of regulation should be given to identification of estrogenic potency of (unknown) compounds in sediments, in contrast to the possibly unwarranted widespread focus on the estrogenic effects of compounds such as phthalates which demonstrated minimal estrogenic potency *in vitro* and *in vivo*.

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Samenvatting

Sinds het begin van de jaren 1990 is in toenemende mate melding gemaakt van endocriene stoornissen bij zowel de mens als organismen in het natuurlijke milieu. Geconstateerde verschijnselen omvatten o.a. verminderde spermakwaliteit bij mannen en misvormingen van de geslachtsorganen en seksuele gedragsstoornissen bij dieren in het wild. Deze verontrustende effecten zouden mogelijk te wijten zijn aan de aanwezigheid van stoffen in het milieu die o.a. de werking van oestrogenen, vrouwelijke geslachtshormonen, mogelijk kunnen nabootsen of remmen. Deze meldingen hebben aanleiding gegeven tot veel wetenschappelijke onderzoek om het verband te zoeken tussen negatieve reproductie effecten bij mannelijke organismen en blootstelling aan (xeno-)oestrogene stoffen. Bovendien werd geadviseerd door nationale en internationale beleidsorganen om nieuwe en bestaande chemicaliën te testen op oestrogene werking. Omdat stoffen met (anti-)oestrogene activiteit uiteenlopende chemische structuren hebben, is identificatie op basis van de chemische structuur moeilijk. Bestaande biologische testmethoden, zogenoemde "bioassays" bleken onvoldoende om snel specifieke effecten van (xeno-)oestrogenen te bepalen. Het werd duidelijk dat het ontwikkelen van snelle bioassays om stoffen te testen op oestrogeen verstorende potentie noodzakelijk was.

Het doel van het in dit proefschrift beschreven onderzoek was om nieuwe *in vitro* en *in vivo* bioassays te ontwikkelen om (xeno-)oestrogene stoffen te detecteren op basis van hun biologisch werkingsmechanisme. Dit werkingsmechanisme berust op het binden van oestrogenen aan oestrogeen receptoren (ER) in de cel en het induceren van ER-gemedieerde gen transcriptie en translatie. Deze nieuwe bioassays beoogen om op gevoelige wijze de blootstelling van biologisch actieve (xeno-)oestrogene stoffen in het aquatische milieu in Nederland te bepalen, en bovendien de mogelijke effecten van blootstelling aan deze stoffen op de reproductie of endocriene functies van vissen te voorspellen.

Er zijn twee nieuwe testsystemen ontwikkeld die gebruik maken van een reporter gen, in dit geval coderend voor het lichtproducerende luciferase afkomstig van het vuurvliegje. Allereerst is een *in vitro* reporter gen assay ontwikkeld. Deze assay gebruikt een menselijke borst kanker cellijn waarin een oestrogeen-induceerbaar luciferase reporter gen op stabiele wijze tot expressie komt. Op basis van deze cellijn is de zogenaamde ER-CALUX (Estrogen Receptor-mediated Chemical Activated LUciferase gene eXpression) assay ontwikkeld om snel (mengsels van) stoffen uit waterige en vaste milieufracties te kunnen testen op (anti-)oestrogene potentie. Naast de ER-CALUX assay is een tweede biologisch testsysteem ontwikkeld dat gebruik maakt van transgene zebravissen. In deze *in vivo* reporter gen bioassay kan de expressie van hetzelfde oestrogeen gereguleerde luciferase reporter gen in doelorganen bepaald worden. Tevens kunnen de kritieke levensstadia van de zebravis bepaald worden voor blootstelling aan vermoede (xeno-)oestrogene stoffen. Hieronder worden de resultaten behaald met beide assays samengevat.

In vitro reporter gen assay (ER-CALUX)

De ER-CALUX assay maakt gebruik van de T47D menselijke borst kanker cellijn stabiel voorzien van een recombinant gen coderend voor het luciferase eiwit. Het ontwikkelen van deze cellijn wordt in Hoofdstuk 2 beschreven. De ER-CALUX methode is gebaseerd op het werkingmechanisme van oestrogenen in levende cellen: oestrogenen binden aan de endogene ER's in de T47D cel, waardoor de receptor wordt geactiveerd en bindt aan specifiek DNA sequenties (de 'estrogen response elements' of ER's) op het luciferase gen. Blootstelling van deze cellen aan (xeno-)oestrogenen resulteert in de vorming van luciferase. Luciferase is heel gevoelig in cel lysaten te meten omdat het na toevoegen van het substraat luciferine, licht wordt geproduceerd.

De respons van de ER-CALUX assay is gekarakteriseerd met diverse (mengsels van) oestrogene en anti-oestrogene stoffen (Hoofdstukken 2 en 3). De assay blijkt uiterst gevoelig en heeft in microtiter platen voor het natuurlijke oestradiol (E2) een detectie limiet van 0.5 pM, een EC50 van 6 pM en maximale inductie van ca. 75-voud bij 30 pM E2. Anti-oestrogenen zoals ICI 182,780, tamoxifen en TCDD kunnen in de ER-CALUX gedetecteerd worden door co-administratie met E2. Een scala aan xeno-oestrogenen waaronder alkylfenolen en alkylfenol-ethoxylaten, organochloor pesticiden, ftalaten en fyto-oestrogenen zijn getest in de ER-CALUX en bleken 1000-100000 maal minder potent dan E2. Genisteine, nonylfenol en o,p'DDT waren van de geteste stoffen de meest potente xeno-oestrogenen (EC50 100, 260 en 620 nM, resp.). De ER-CALUX assay is ook gebruikt om de interactie

tussen verschillende combinaties van stoffen te onderzoeken. Over het algemeen bleken combinaties van organochloor pesticiden additiviteit te vertonen (Hoofdstuk 2). Om na te gaan of metabolieten van (xeno-)estrogene stoffen zelf ook een oestrogene werking konden vertonen, is een *in vitro* biotransformatie stap aan de ER-CALUX assay procedure toegevoegd. Hiertoe zijn uitgangstoffen vooraf geïncubeerd met microsomen van P450 geïnduceerde rattenlevers. De metabolieten van de xeno-oestrogeen methoxychloor bleken een hogere oestrogene activiteit te vertonen dan de uitgangsstof (Hoofdstuk 3).

De respons in de ER-CALUX assay is vergeleken met een andere veel gebruikte reporter gen assay voor oestrogene werking, de zogenoemde "recombinant yeast screen". Deze assay maakt gebruik van gist-cellen die voorzien zijn van een recombinante menselijke oestrogeen receptor en een ER-gemedieerd β-galactosidase reporter gen. Vergelijking tussen de ER-CALUX assay en de gist assay laat zien dat de ER-CALUX assay E2, nonylfenol en o,p'DDT detecteert bij een 10-100 keer lagere concentratie dan de gist assay (Hoofdstuk 3). Experimenten met anti-oestrogenen laten zien, dat de ER-CALUX assay ook veel gevoeliger is voor anti-oestrogenen dan de gist assay (Hoofdstuk 3).

De hoge gevoeligheid van de ER-CALUX assay en mogelijkheid om een relatief hoge aantal monsters op een snelle manier te testen maakt het een ideale methode om oestrogene activiteit aan te tonen in milieumonsters. Onderzoek naar de optimale extractie methode voor waterbodems liet zien dat relatief polaire componenten in hexaan:aceton extracten hogere oestrogene activiteit bevatten dan apolaire componenten (Hoofdstuk 3). Sediment extracten van geïndustrialiseerde locaties, zoals de haven van Rotterdam vertoonden een verhoogde oestrogene potentie vergeleken met referentie gebieden (Hoofdstuk 3). Naast sediment is in deze studie de oestrogene activiteit gemeten in oppervlaktewater, zwevend stof en biota (mosselen en vissen) in verschillende locaties in Nederland (Hoofdstuk 4), en van effluent van een huishoudelijke rioolwaterzuiveringsinstallatie (Hoofdstuk 6).

Er is ook een methode ontwikkeld om te bepalen of biologische inactieve (xeno-) oestrogene metabolieten aanwezig in humane urine en vissengal opnieuw geactiveerd kunnen worden (Hoofdstuk 4). Urine bleek na voorbehandeling met het enzym β-glucuronidase of *E. coli* cellen weer actieve uitgangsstoffen te bevatten waardoor ze een secundaire bron van oestrogene stoffen kunnen vormen in het milieu. Ook in galmonsters van vissen zijn geconjugeerde metabolieten aanwezig die na een dergelijke deconjugatie weer oestrogene potentie bevatten waardoor ze eveneens een secondaire bron van oestrogenen kunnen vormen in de vis. Hoge oestrogene activiteit van gedeconjugeerde gal monsters van mannelijke brasem van verschillende locaties in Nederland correleerde met verhoogde oestrogene activiteit in water en vitellogenine inductie in plasma van dezelfde vissen (Hoofdstuk 4). De oestrogene potentie in (gedeconjugeerde) metabolieten van gal en urine is een veelbelovende biomarker voor de interne dosis van (xeno-)oestrogenen in mannelijke organismen.

In vivo reporter gen assay (transgene zebravissen)

Transgene zebravissen zijn ontwikkeld met hetzelfde oestrogeen-gevoelige luciferase reporter gen als de ER-CALUX assay stabiel geïntegreerd in hun genoom (Hoofdstuk 5). Dit is de eerste melding van een *in vivo* reporter gen assay om de blootstelling van (xeno-) oestrogenen te bepalen. Luciferase reporter gen expressie is in deze assay niet alleen afhankelijk van de bindingscapaciteit van oestrogenen aan endogene ER's maar ook van de biologische beschikbaarheid en *in vivo* toxicokinetiek van de stof.

De transgene zebravissen zijn gegenereerd met een effectieve methode dat gebruik maakt van microinjectie van DNA constructen in eencellige embryo's. De ontwikkelde stabiele transgene lijn vertoont hoge luciferase expressie na blootstelling aan E2. Luciferase expressie is gevolgd tot de vijfde generatie van deze lijn, en is stabiel gebleven. De gevoeligheid van de transgene zebravissen voor E2 bleek sterk afhankelijk van het levensstadium. De hoogste luciferase inductie tijdens de juveniele ontwikkeling is gevonden in vissen van 4-5 weken oud, de periode waarin gonadendifferentiatie plaatsvindt. Interessant is dat ook zeer jonge embryo's (24 uur oud) al actieve oestrogeen receptoren bevatten. In transgene vissen van 4-5 weken oud leidde blootstelling aan zowel natuurlijke oestrogenen (17α-oestradiol, oestron) als het synthetische oestrogeen ethinyloestradiol (EE2) en het xeno-oestrogeen o,p'DDT tot verhoogde luciferase activiteit. In volwassen transgenen bleken bij kortstondige blootstelling aan E2 lever, spier, schubben en botten potentiële doelorganen voor oestrogenen. In de testes van volwassen mannetjes vertoonden de hoogste waargenomen luciferase activiteit van minimaal 1000 maal na 48 uur blootstelling aan 1 μM E2. Immunohistochemie op testis coupes liet zien dat luciferase expressie gelokaliseerd is in vroege kiemcellen en Sertoli cellen (Hoofdstuk 5).

Omdat luciferase expressie in de transgene vissen afhankelijk is van endogene ER niveaus, is van twee ER-vormen de expressie in de zebravis bestudeerd. De ER-vormen α en β zijn gedetecteerd in zeer jonge embryos (24 uur oud) en komen sterk tot expressie in de periode van geslachtsdifferentiatie. In volwassen vissen bleken weefsels met hoge luciferase activiteit (testis, lever) zowel ER-α als ER-β te bevatten (Hoofdstuk 5).

De responsen in de *in vitro* ER-CALUX assay en de *in vivo* transgene zebravis assay zijn vergeleken na blootstelling aan (mengsels van) (xeno-)oestrogenen (Hoofdstuk 6). Beide assays zijn gevoelig voor natuurlijke (E2 en oestron) en synthetische (ethinyloestradiol, EE2) oestrogenen, maar er waren duidelijke verschillen in relatieve potenties ten opzichte van E2 zijn waargenomen. EE2 was 100 keer potenter dan E2 in de transgene vissen, maar heeft ongeveer dezelfde potentie als E2 in de ER-CALUX assay. De bioaccumulerende xeno-oestrogeen o,p'DDT liet vergelijkbare relatieve potenties in beide assays zien. De snel metaboliseerbare xeno-oestrogenen nonylfenol en bisfenol A gaven echter verhoogde oestrogene activiteit *in vitro*, maar induceerden geen reporter gen activiteit *in vivo* (Hoofdstuk 6). Deze resultaten tonen aan dat stabiele stoffen *in vivo*, zoals EE2 en o,p'DDT, hogere oestrogene activiteit kunnen vertonen dan voorspeld wordt met *in vitro* bioassays. Deze stoffen verdienen extra aandacht bij de risicobeoordeling van (xeno-)oestrogenen voor aquatische organismen terwijl snel metaboliseerbare stoffen wellicht een mindere bedreiging vormen dan gedacht.

In een vergelijkend onderzoek zijn beide assays toegepast op extracten van effluent van een Nederlandse zuiveringinstallatie voor huishoudelijke rioolwater. Dit effluent is in detail onderzocht omdat in eerdere studies in het ontvangende rivierwater en galmonsters van mannelijke vissen hoge oestrogene activiteit werd gemeten (Hoofdstuk 4). Bovendien bevatten deze vissen verhoogde plasma vitellogenine waarden (Hoofdstuk 4). De oestrogene potentie in dit effluent was hoger in de transgene zebravis assay dan de *in vitro* assay, waarschijnlijk door de aanwezigheid van het *in vivo* zeer potente EE2. Er werd ca. 200 pmol E2 equivalenten per liter effluent gemeten na 96-uur blootstelling van juveniele transgene zebravissen. Deze concentratie overschrijdt effectwaarden gerapporteerd in literatuur voor reproductie effecten in vissen. Dit betekent derhalve dat vissen in deze locatie mogelijk blootgesteld zijn aan niveaus van (xeno-)oestrogenen die hun reproductieve succes zouden kunnen beïnvloeden.

Het onderzoek beschreven in dit proefschrift leidt tot de conclusie dat de ER-CALUX en transgene zebravis assays veelbelovende *in vitro* en *in vivo* assays zijn voor blootstelling aan en potentiële effecten van stoffen met een oestrogene werking. De ER-CALUX assay is snel en makkelijk uit te voeren, en kan toegepast worden voor het kwantificeren van complexe milieumonsters voor oestrogene activiteit. De transgene zebravis assay is de ideale aanvulling voor de *in vitro* assay, omdat deze ook de beschikbaarheid en toxicokinetiek van stoffen *in vivo* integreert. Deze assay biedt een unieke, snelle methode om de effecten te bepalen van (xeno-)oestrogenen op doelorgangen en kritieke levensstadia in de ontwikkeling van de zebravis. Op basis van de nu bekende relatieve oestrogene potentie van stoffen *in vivo* is het mogelijk de voorspelende waarde van de *in vitro* assay te bepalen. Daardoor kunnen beide assays worden ingezet voor het determineren of stoffen in het milieu aanwezig zijn bij concentraties die kunnen leiden tot effecten op reproductie of endocriene functies in aquatische organismen. Met behulp van deze technieken zijn in Nederland op enkele plaatsen al niveaus van biologische active (xeno-)oestrogenen gevonden die zeer waarschijnlijk ongewenste effecten op de voortplanting van vissen kunnen veroorzaken.

Curriculum vitae

Juliette Legler was born in Brampton, Ontario, Canada on December 29, 1969. She graduated with an American secondary school diploma from the Colegio F.D. Roosevelt, Lima, Peru in 1986 and with a Canadian secondary school diploma from Brampton Centennial Secondary School in 1987. In 1987 she commenced studies at the University of Waterloo, Canada and graduated in 1992 with an Honours Bachelor's degree in Environmental Studies (minor in Biology). During this study she carried out work terms at the Canadian International Development Agency and Environment Canada. During a one-year exchange program in the Netherlands from 1990-1991, she worked at the Ministry of Public works, Institute for Coastal and Marine Management (RWS/RIKZ) in Middelburg, under the supervision of Ir. J. Stronkhorst. She also conducted toxicological research at the RIKZ's Jacoba Haven Field Station (supervisor: Dr. P. van den Hurk). From 1991-1992, she performed research at the University of Waterloo's Aquatic Toxicology Laboratory under the supervision of Dr. P. Sibley. In 1992 she commenced studies in the Netherlands at the Wageningen University (WU) and graduated in 1994 with a Master's of Environmental Sciences degree. During this study she carried out a 10-month research project at the Department of Toxicology (supervisor: Dr. A.J. Murk) in collaboration with the Dutch National Institute for Inland Water Management (RWS/RIZA) (supervisor: Dr. C. van den Guchte). In 1995 she was employed as a researcher at the Department of Toxicology (WU), and commenced her PhD studies in 1996. Her PhD project, performed under the auspices of RWS/RIKZ, was a collaboration between the Department of Toxicology, WU (Dr. A.D. Murk) and the Hubrecht Laboratory (Netherlands Institute for Developmental Biology, Dr. B. van der Burg). The research presented in this thesis was conducted between 1996 and 2000. Since February 2000 she has been employed as a post-doctoral researcher at the Hubrecht Laboratory in collaboration with the Department of Toxicology, WU.

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