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No. 135

DETAILED REVIEW PAPER ON ENVIRONMENTAL ENDOCRINE DISRUPTOR SCREENING: THE USE OF ESTROGEN AND ANDROGEN RECEPTOR BINDING AND TRANSACTIVATION ASSAYS IN FISH

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No. 135

Detailed Review Paper on Environmental Endocrine Disruptor Screening: The use of Estrogen and Androgen Receptor Binding and Transactivation Assays in Fish

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FOREWORD

The development of this Detailed Review Paper (DRP) on "*Environmental Endocrine Disruptor Screening: The use of Estrogen and Androgen Receptor Binding and Transactivation Assays in Fish*" was initiated in 2004 and has been led by Japan, Sweden and the United Kingdom. The assays covered by the DRP were intended for the level 2 of the Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals, "*In Vitro Assays Providing Mechanistic Data*".

At a meeting of the Validation Management Group for Non-Animal testing (VMG NA) in 2004, the International Council for the Protection of Animals in OECD Programmes (ICAPO) raised the issue of the development of a Detailed Review Paper (DRP) on "*In Vitro* Vitellogenin Assays".

The VMG NA considered this issue to be more appropriately handled by the VMG for Ecotoxicity testing (VMG-eco) and the Secretariat raised the topic at the VMG-eco meeting that was held in Paris on 8-9 December 2004 and explained the need of expertise from the VMG-eco to develop this DRP. The VMG eco agreed to the proposal and an Expert Group was established in April 2005.

A first draft was presented to the Working Group of National Coordinators of the Test Guidelines Programme (WNT) in 2006 and since there were some issues raised on the scope of the DRP, the WNT agreed to focus the DRP more on fish *in vitro* estrogen and androgen receptor binding and transcriptional activation assays. A new Standard Project Submission Form was submitted from the lead countries Japan, Sweden and the United Kingdom.

A revised draft DRP was made available to the VMGs NA and eco in the 3rd quarter of 2007 and a Fish *In Vitro* subgroup of the VMG NA further edited the draft before re-submitting it toVMG-eco. The document was then further edited by the Secretariat. The draft DRP was approved by the WNT on 2 July 2010 by written procedure, and the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 15 September 2010.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

TABLE OF CONTENTS

ABBREVIATIONS

11-KT:	11-ketotestosterone
4-HT:	4-hydroxytamoxifen
ADME:	Absorption, Distribution, Metabolism and Excretion
AHTN:	6-acetyl-1,1,2,4,4,7-hexamethyltetraline
AR:	Androgen Receptor
as:	Atlantic salmon
asER:	Atlantic salmon Estrogen Receptor
at:	Atlantic croaker
at ER:	Atlantic croaker Estrogen Receptor
B_{max}:	Binding capacity
BBP:	Butylbenzyl Phthalate
BP:	Benzophenone
BPA:	Bisphenol A
CALUX:	Chemically Activated LUciferase eXpression
cc:	channel catfish
ccER:	channel catfish Estrogen Receptor
ccSHBG:	channel catfish Sex Hormone-Binding Globulin,
Cd:	Cadmium
ChgH:	Choriogenin H
CR:	Cortisol Receptor
DBHP:	Di-*n*-Buthylhexyl Phthalate
DBP:	Di-*n*-Butyl Phthalate
DDA:	Bis(*p*-chlorophenyl)acetic Acid
DDD:	Dichloro-Diphenyl-Dichloroethane
DDE:	Dichloro-Diphenyl-dichloroEthylene
DDT :	Dichloro-Diphenyl-Trichloroethane
DEHP:	Di(2-Ethylhexyl) Phthalate
DES :	Diethylstilbestrol
DHT:	Dihydrotestosterone
DNA:	Deoxyribonucleic Acid
DRP:	Detailed Review Paper
E1:	Estrone
E2:	17β-Estradiol
E3:	Estriol
EC_{50}:	Effective concentration for 50% maximal response
EDSP:	Endocrine Disruption Screening Program
EDTA:	Endocrine Disrupters Testing and Assessment
EE2:	17α-Ethynylestradiol
EPC:	*Epithelioma Papulosum Cyprini*
ER:	Estrogen Receptor
ERE:	Estrogen Responsive Elements
ESTs:	Expressed Sequence Tags

fhERα:	*Fundulus heteroclitus* Estrogen Receptor-α
fhm:	Fathead minnow
fhmAR:	Fathead minnow Androgen Receptor
fhmER:	Fathead minnow Estrogen Receptor
GC/MS:	Gas Chromatography-Mass Spectrometry
GEN:	Genistein
GFP:	Green Fluorescence Protein
GST:	Glutathione *S*-Transferase
hAR:	human Androgen Receptor
hER	human Estrogen Receptor
hGR:	human Glucocorticoid Receptor
HHCB:	1,2,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-gamma-2-benzopyran
HPG:	Hypothalamic-Pituitary-Gonadal
IC_{50}:	Half maximal inhibitory concentration
jgs:	Japanese giant salamander
jgsER:	Japanese giant salamander Estrogen Receptor
K_d:	Dissociation constant
LBD:	Ligand Binding Domain
LOEC:	Lowest-Observed-Effect Concentrations
mER:	Medaka Estrogen Receptor
mAR:	Medaka Androgen Receptor
mqfAR:	Mosquitofish Androgen Receptor
mqfER:	Mosquitofish Estrogen Receptor
MMTV:	Mouse Mammary Tumor Virus
mRNA:	messenger Ribonucleic acid
MT:	Methyltestosterone
MXC:	Methoxychlor
NP:	NonylPhenol
OCS:	Octachlorostyrene
OP:	4-*t*-Octylphenol
P:	Progesterone
PCB:	Poly-Chlorinated Bi-phenyls
PCR:	Polymerase Chain Reaction
PME:	Pulp-Mill Effluent
PR:	Progesterone Receptor
RACE :	Rapid Amplification of cDNA
RBA:	Relative Binding Affinity
rbt:	Rainbow trout
rbtAR :	Rainbow trout Androgen Receptor
rbtER :	Rainbow trout Estrogen Receptor
qRT-PCR:	quantitative Reverse Transcription Polymerase Chain Reaction
SBP :	Sex Steroid-Binding Protein
SHBG:	Sex Hormone-Binding Globulin
ss :	Spotted sea trout
ssER:	Spotted sea trout Estrogen Receptor
T :	Testosterone
TA :	Transcriptional Activation Assays
TAM:	Tamoxifen
TB:	Trenbolone
TBT:	Tributyltin chloride
TR:	Thyroid hormone receptor

US EPA:	US Environmental Protection Agency
VMG:	OECD Validation management Group
VTG:	Vitellogenin
YES:	Yeast Estrogen System
ZEA:	Zearalenone
zf:	Zebrafish
zfER:	Zebrafish Estrogen Receptor
ZR:	*Zona Radiata*

I. INTRODUCTION

1. The current Detailed Review Paper (DRP) has been developed under the Validation Management Groups for Ecotoxicity Testing (VMGeco) and Non-Animal testing (VMG NA). The DRP focuses on estrogen and androgen *in vitro* receptor and transcriptional activation (TA) assays available for fish species and does not attempt to include additional wildlife taxa such as mammals, amphibians, reptiles, birds, or the invertebrates. The assays covered by the DRP are primarily for the level 2 of the Conceptual Framework of the Endocrine Disrupters Testing and Assessment Task force (EDTA), "*In Vitro* Assays Providing Mechanistic Data", with the exception of the transgenic fish model systems.

2. Across many aspects of endocrine disrupter research, *in vitro* assays have proven to be of significant value in screening for intrinsic endocrine activity and also for mechanistic research studies. Yeast based assays and mammalian cell lines have been widely used for both biomedical (Körner *et al.,* 2004; Soto *et al.,* 2006) and environmental applications (Routledge and Sumpter, 1996; Desbrow *et al.,* 1999; Beck *et al.,* 2006). However, *in vitro* assays based on fish tissues also hold significant promise as a tool for use in tiered testing strategies of endocrine activity (Navas and Segner, 2006). Identification of nuclear receptor-mediated endocrine activities is important in a variety of fields, ranging from pharmacological and clinical screening, to food and feed safety, toxicological monitoring, and risk assessment. Traditionally animal studies such as the Hershberger assay and the Allen-Doisy or the Uterotrophic assay have been used for the assessment of androgenic and estrogenic potencies, respectively. To allow fast analysis of the activities of new chemicals, food additives, and pharmaceutical compounds, high-throughput screening strategies have been developed.

3. A wide range of organic contaminant compounds prevalent in the aquatic environment has been shown to exhibit hormone-disrupting activity. The actual potency of such compounds is low compared with endogenous hormones, such as 17β-estradiol (E2), but may still produce detrimental biological effects (Barnes *et al.,* 2008; Focazio *et al.,* 2008). Field and laboratory studies on the biological effects of environmental estrogens have, in the past, largely relied on assays of vitellogenin (VTG) induction in male fish (Scholz *et al.,* 2004; Eidem *et al.,* 2006; for review, Iguchi et al., 2006a), reduced growth in testes formation, and intersex incidence (Tyler *et al.,*1998). Fish hepatocytes have also been successfully used as an *in vitro* system for screening intrinsic endocrine activity based on VTG measurement (Pelissero *et al.,* 1993; Rankouhi *et al.,* 2004; Navas and Segner, 2006).

National and International Activities

The EDTA and the three VMGs

4. At the request of member countries and the international industry, OECD initiated in 1997 the Special Activity on Endocrine Disrupters Testing and Assessment with the objectives to provide a set of internationally recognised and harmonised Test Guidelines and testing and assessment strategies for regulatory use that would avoid duplication of testing and thus save resources, including animals.

5. Managed by the Endocrine Disrupters Testing and Assessment Task Force (EDTA) and its three Validation Management Groups on mammalian tests (VMG-mammalian) on ecotoxicity tests (VMG-eco) and on non-animal tests (VMG-NA), several comprehensive test validation projects have been completed. The first objective was to identify and prioritize development of new and updating of existing Test Guidelines. A conceptual framework was agreed in 2002 and it is composed of five levels ranking different types of *in silico, in vitro* and *in vivo* assays, to be used according to countries regulatory needs. The

validation of a number of test methods on biotic systems and health effects then started under the leadership of three VMGs. The OECD Guidance Document 34 (2005) provides 8 criteria that should be considered in the validation of test methods.

6. The VMG NA have approximately 20 *in vitro* assays under development and the TG 455 on the "*Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (STTA)",* was the first Test Guideline from the VMG NA to be adopted, in 2009 (OECD, 2009a). Other projects under validation or in peer review include receptor binding and TA assays for human ERα, AR assays and a H295R Steroidogenesis assay. The Test Guideline 440 for the Uterotrophic Bioassay, a screening assay for estrogenic effects in rodents, was adopted in 2007 (OECD 2007). The Test Guidelines for the Hershberger assay (OECD 2009b), the updated Test Guideline 407 (Repeated dose 28-day oral toxicity) (OECD 2008) and the Test Guideline for the 21-day Fish Screening Assay (OECD 2009c) have all been approved. About 25 projects of the work plan for the Test Guideline Programme 2006/8 are related to the development of Test Guidelines or related documents for endocrine testing. The OECD Validation Management Group for Ecotoxicity Testing (VMG-eco) is establishing screening and testing programs for endocrine disrupting chemicals. For fish, 21-day exposure assay including VTG induction and alteration of apical endpoints and gonad histology have been conducted to detect estrogenicity, anti-estrogenicity, androgenicity, anti-androgenicity and aromatase activity of chemicals using fathead minnow (*Pimephales promelas*), zebrafish, medaka and stickleback. Amphibian metamorphosis assay using stage 51 *Xenopus lavevis* tadpoles has been conducted to detect thyroid active and anti-thyroid hormone activity including disruption of deiodination. For invertebrate species, mainly lifecycle (including development and reproduction) test methods using chironomids, copepods, daphnids and mysids are under discussion. For avian, a two-generation test using Japanese quail (*Coturnix japonica*) is under discussion by the avian expert group.

National Activities

7. The US Environmental Protection Agency (US EPA) Endocrine Disruption Screening Program (EDSP) has developed and validated *in vitro* and *in vivo* assays to determine the potential for chemicals to cause endocrine disruption in humans or wildlife [http://www.epa.gov/scipoly/oscpendo/index.htm]. US EPA is using a two-tiered approach. The Tier 1 Screening battery is based on the Endocrine Disruptor Screening and Testing Advisory Committee's (EDSTAC) recommendations and is intended to identify chemicals potentially affecting the estrogen, androgen, or thyroid hormone systems through any of several recognized modes of action across mammalian and ecological taxa. Tier 2 Testing is intended to confirm, characterize, and quantify those effects for estrogen, androgen, and thyroid active substances.

8. The Tier 1 Screening battery includes *in vitro* mammalian estrogen receptor (ER) and STTA assays, an androgen receptor (AR) binding assay, a steroidogenesis assay using H295R cells, and a recombinant aromatase assay. There are no *in vitro* fish or amphibian assays included in the battery, but *in vivo* fish and amphibian assays are included. The fathead minnow assay can identify endocrine disrupting chemicals displaying several mechanisms of concern, including AR and ER agonists and antagonists and inhibitors of steroid hormone synthesis (Ankley and Villeneuve, 2006). An amphibian metamorphosis assay will detect thyroid-active substances (Gray *et al.*, 2002; Degitz *et al.*, 2005). Additional *in vivo* assays include the mammalian utertrophic assay for estrogen agonist, the Hershberger for androgen and anti-androgens, the male pubertal assay for androgens, anti-androgens and thyroid active agents and HPG and HPT axis disruption, and the female pubertal for estrogen and anti-estrogens and thyroid active agents as well as HPG and HPT axis disruption.

9. The Ministry of Environment (MoE) of Japan conducted the VTG assay, partial life cycle test and full life cycle test using medaka for detecting estrogenicity of chemicals and adverse effects of chemicals. Receptor binding assay (Nakai, 2003) and reporter gene assay using medaka ERs have been established. A

Daphnia magna assay has been successfully used to detect juvenile hormone-like effect and ecdysone-like effect of chemicals. *Daphnia magna* ecdysone reporter gene assay has been established (Kato *et al.*, 2007). Currently, microarray systems of medaka and *Daphnia magna* are under construction (Watanabe et al., 2007). Metamorphosis assays using *Xenopus laevis* and *Silurana tropicals* have been conducted (Opitz *et al.*, 2005; Mitusi *et al.*, 2006).

10. The Danish Environmental Protection Agency evaluated *in vitro* assays for determination of estrogenic activity in the environment such as ER binding assay, ER reporter gene assay and cell proliferation assay using MCF-7 breast cancer cells (Kinnberg, 2003).

Introduction to test systems

11. Assays for fish ER, AR and progesterone receptor (PR) and invertebrate ecdysone receptor, VTG, steroid biosynthesis enzymes (aromatase, sulfotransferase and hydroxysteroid dehydrogenase) may be useful tools to screen chemicals for their endocrine activity and to develop a mechanism-based integrated testing strategy for further investigations of higher tier test methods. Molecular methods of gauging VTG and *zona radiata* (ZR) expression and protein concentrations have included immunoassay and quantitative polymerase chain reaction (PCR). The isolation of key gene expression products (*e.g.*, aromatase, ZR, VTG) from a wider range of fish species is essential. Endocrine disruption in invertebrates has received less attention compared with fish, partly because knowledge regarding invertebrate endocrinology is limited (Hutchinson, 2002, 2007; Rotchell and Ostrander, 2003). Recently, Expressed Sequence Tags (ESTs) of *Daphnia magna* have been read and clustered (Watanabe *et al.,* 2005). In the future, more reliance will be placed on the development of gene expression assays using reporter systems combined with PCR-based assays, or even microarray systems (Iguchi *et al.,* 2006b, 2007) and proteomics approaches as they arise from biomedical and environmental research efforts (Link *et al.,* 2006).

12. Most current assays for measuring endocrine activity use mammalian systems; however, extrapolations of potential hazard to other vertebrates based on mammalian data could be over- or under-protective. The conservation of many aspects of endocrine function among vertebrate species (Norris, 1996; Van Der Kraak *et al.*, 1998) provides a biological basis for extrapolating what is known about effects of chemicals on endocrine activity between species (Kavlock and Ankley, 1996). Uncertainties associated with such extrapolations include a lack of comparative knowledge about basic processes such as ligand-receptor binding. For example, although there is structural homology among ERs from different species, specific structural and functional differences between the rainbow trout ERs (rbtERs) and mammalian ERs have been reported. Specifically, Petit *et al.* (1997) reported that there was 60% homology between the ligand-binding domain of rbtERα and human ERα (hERα). The E2 concentration required to achieve maximal binding in a yeast expressed receptor (Matthews *et al.,* 2000) was 10-fold higher for the rbtER than that for other species' ERs, including hER. In rbtERα, the 50% maximal effective concentration (EC_{50}) values for E2 at 37°C were 1/28 of that at 20°C in MCF-7 breast cancer cells (Matthews *et al.*, 2002), which indicates the importance of assay temperature to achieve maximal transactivation. Structural homology among ERs from various animal species has been summarized in Katsu *et al.* (2006a, 2008). The extent to which such differences would affect quantitative extrapolation of chemical binding affinity between vertebrate classes or among species within a class, is not clear.

13. This DRP summarizes the use of receptor binding assays using extracted ER and AR proteins and recombinant receptors as a means of primary screening of environmental samples and chemicals for estrogenicity, anti-estrogenicity, androgenicty, and anti-androgenicity in fish species, which avoids species and seasonal variation in receptor response to ligand binding. Transcriptional activation (TA) assays using native full-length ER and AR, and engineered ER and AR systems containing transfected receptor genes

with reporter plasmid, have been established for various fish species and will also be summarized (Tables 1 and 2). In these assay systems, both agonistic and antagonistic activities of chemicals can be measured without using radio-isotopes. In addition, ER and AR genes from various fish species can be easily transfected into cell lines and provide standard base-line when the same cell line is used.

II. DESCRIPTION OF METHODS

Receptor Binding Assays

14. Competitive ligand binding assays are used to gain mechanistic insight into the primary mode of action of xenoestrogens or xenoandrogens by determining their affinity for ER or AR, respectively. *In vitro* competitive binding assays for the ER have been well established and extensively used to investigate ER-ligand interactions. ER binding assays can be performed with receptors obtained from a) endogenous sources, *i.e.*, cytosolic or nuclear extracts of various tissues (*e.g.*, liver, gonads), or b) recombinant ER full length receptor or Ligand Binding Domain (LBD) fusion proteins prepared using *Eschelichia coli* (*E. coli*) or insect cells and obtained from cell extracts. Most ER binding assays quantify the ability of a test compound to compete with radio-labelled E2, [^{3}H]-E2, for binding to the ER. In a typical competitive hormone binding assay, cytosol, cell extract or recombinant ER LBD fusion protein prepared using *E. coli* is incubated with [^{3}H]-E2 and various concentrations of unlabelled test compounds. If compounds compete with the [^{3}H]-E2 for receptor binding, they will displace a fraction of the [^{3}H]-E2 from the receptor in a concentration dependent manner. As the concentration of the unlabelled competitor increases, more [^{3}H]-E2 is displaced from the ER. Usually, the free [^{3}H]-E2 is separated from the bound [^{3}H]-E2 by dextran-coated charcoal or hydroxyapatite treatments or other methods and receptor-bound [^{3}H]-E2 is quantified by liquid scintillation counting. Non-specific binding is measured by addition of excesses of radio-inert diethylstilbestrol (DES) or E2. The specific binding of [^{3}H]-E2 to the ER is calculated by subtracting the amount of non-specific binding from the amount of [^{3}H]-E2 bound in the absence of a competitor. Decreased specific binding of the [^{3}H]-E2 in the presence of a test sample suggests that the sample contains compounds that can competitively bind to ER ligand-binding site. Determinations of ER binding abilities do not classify the ligand as agonist or antagonist. The ability of a substance to initiate the molecular cascade of events implicated in gene transcription and protein synthesis associated with adverse effects can not be determined in this assay.

Transcriptional Activation Assays (TA)

15. The ER functions as a modulator of the transcription of its target cell genes. ER TA assays are based on the ability of a compound to stimulate ER-dependent gene transcription. Thus, transactivation is a result of the molecular cascade of events initiated by receptor occupation and completed by nuclear binding and transactivation of a reporter gene, and as such provides an indication of the estrogenic activity of a compound at a higher level of biological organization than receptor binding alone.

16. Fish *in vitro* TA assays can be carried out using a) endogenously-derived systems such as primary hepatocytes or liver slices, or b) genetically engineered systems similar to those used with mammalian receptors. Fish primary hepatocyte and liver slice transactivation assays take advantage of the fact that male fish livers possess ER and the capacity to produce VTG, yet normally the egg yolk precursor protein is only synthesized in the livers of female oviparous vertebrates in response to estrogenic stimulation. In these systems, estrogenic effects of xenobiotics can be easily measured at the protein level by observing VTG levels, but also at the transcriptional level by observing the induction of any estrogen dependent gene, such as those of VTG or the same ER. Antagonism is also detected by co-treating with E2 and chemical. Isolated fish hepatocytes (Navas and Segner, 2006) and liver slices (Shilling and Williams, 2000; Schmieder *et al.,* 2000, 2004) (Table 2) have been effectively used to screen for anti-estrogenic activity of

chemicals based on the reduction in the production of E2-induced VTG.

17. Systems genetically engineered with recombinant fish receptors can be derived using mammalian or fish cell lines, or strains of yeast (Ackermann *et al.*, 2002; Paris *et al.*, 2002; Pillon *et al.*, 2005). In the genetically engineered systems, the cells are transfected by introducing vectors containing DNA sequences for the receptor, along with Estrogen Responsive Elements (EREs) linked to a reporter gene, and the reporter gene itself. A number of mammalian-based assays are available using cell lines with an endogenous hER (MCF-7 cells and T47D cells) (Wilson *et al.*, 2004a) or cell lines without an endogenous ER (yeast cells, HeLa cells, CHO cells, etc.). The reporter gene used in human cancer cells usually codes firefly luciferase and the reporter gene used in yeast cells usually codes β-galactosidase. Reporter genes can be introduced into cells for the duration of the experiment only (transient transfection) or permanently (stable transfection). Regardless of whether transient or stably transfected cells are utilized in the assays, test substances incorporated into the cells interact with the ER, which becomes activated by a drastic change in its conformation, especially in Helix 12 (Brzozowski *et al.,* 1997). The ER-ligand complex recruits co-activators, and the resulting complex binds to the ERE on the reporter plasmid, which initiates the transcription of the reporter gene and thereby the production of the reporter enzyme. An appropriate substrate in the incubation mixture is metabolized by the respective newly synthesized enzyme, *e.g.* luciferase or β-galactosidase, and results in the production of an easily detected product.

18. In agonism studies, the cells are treated with a test substance and the induction of the reporter gene products is utilized to measure the response. For an assessment of relative potency, the induction can be compared to the induction by reference estrogen. Alternatively, when dose-response data are generated, the EC_{50} for the test substance can be determined and compared with that for the reference estrogen.

19. For antagonism studies, the cells are exposed simultaneously to the reference estrogen and the test substance, while control cells are exposed to the reference estrogen only. Usually, a constant concentration of reference estrogen is used and the decline of its transcriptional activity with test substances is monitored. The difference in induction of the reporter gene product in the presence and absence of the test substance is used as a measure of estrogen antagonism.

20. Another promising assay system for ER TA has been established by modification of the GAL4 two-hybrid system using mammalian cells (Dang *et al.*, 1991; Fearon *et al.*, 1992). One vector contains the yeast GAL4 DNA-binding domain upstream of a multiple coding region, and expresses *Renilla reniformis* luciferase for normalization of transfection efficiency. The other pG5-luc vector contains five GAL4 binding sites upstream of a minimal TATA-box, which in turn, is upstream of the firefly luciferase gene. Using these two vectors, the luciferase expression in the CHO-K1 cell line can be analyzed. When the GAL4 DNA-binding domain, which was fused with recently cloned Japanese giant salamander (jgs) ERα, and the pG5-luc vector were introduced into CHO-K1 cells, the luciferase expression did not change in the absence of E2 (Katsu *et al.*, 2006b). However, exposure to E2 induced significant expression of luciferase in cells containing the GAL4 DNA-binding domain fused the jgsERα. The latter thus indicates that the GAL4 DNA-binding domain fused with jgsERα can bind to a GAL4 binding site in the pG5-luc vector following the E2-induced change in conformation of the ER, resulting in an increase in the transcription of the firefly luciferase gene. E2 stimulated luciferase production in a dose-dependent manner similar to the ERE-luciferase system (Katsu *et al.*, 2006b). Progesterone did not induce activity of the jgsERα. In addition, the E2-induced luciferase activity in this system was over 50-fold compared to the approximately five-fold activity of the ERE-luciferase system (Katsu *et al.*, 2006b). In order to screen estrogenic, anti-estrogenic, androgenic and anti-androgenic activities of chemicals, the ER and AR binding assays, TA assay and modification of binding assay are available at present. Each assay system has advantages and disadvantages. Current publications of *in vitro* assays using fish ER and AR will be summarized in the following sections.

III. ESTROGEN RECEPTOR

21. Estrogens play important roles in the reproductive biology of vertebrates. The majority of actions of estrogens are mediated by specific receptors that are localized in the nucleus of target cells. ERs belong to a super family of nuclear transcription factors that include all other steroid hormone receptors such as progestogens, androgens, glucocorticoids, mineralocorticoids, Vitamin D receptor, and the retinoic acid receptor (Blumberg and Evans, 1998). Three distinct types of ER have been isolated to date in vertebrates. The teleost ERα, ERβ1 and ER□2-forms but the teleost ER□2-form appears to be closely related to the teleost ERβ1, suggestive of a gene duplication event that occurred within the teleosts (Hawkins *et al.*, 2000). Thus, the ancestral condition for the jawed vertebrates (*Gnathostomata*) is considered to be the presence of two forms of ER, corresponding to ERα and ERβ1 (Thornton, 2001). Indeed, these two forms of ER have been previously found in mammals, fish, birds, reptiles and amphibians. cDNAs encoding for ERα have been cloned from several vertebrate species including mammals (Green *et al*., 1986; Koike *et al*., 1987; White *et al.*, 1987), birds (Krust *et al.*, 1986), reptiles (Sumida *et al.*, 2001; Katsu *et al.*, 2004), amphibians (Weiler *et al.*, 1987) and teleost fish (Pakdel *et al.*, 1990). E2 is the principal estrogen in circulation and appears essential for normal ovarian development in many vertebrates (Wallace, 1985). In chickens and turtles, embryonic exposure to inhibitors of aromatase, the enzyme responsible for the conversion of testosterone (T) to E2, causes genetic females to become phenotypic males (Elbrecht and Smith, 1992; Dorizzi *et al.*, 1994). Likewise, embryonic exposure of various fishes, amphibians or reptiles to E2 or estrogenic chemicals, pharmaceutical agents or environmental contaminants, can induce highly skewed sex ratios toward females (for reviews, see Dietrich and Krieger 2009; Guillette *et al.*, 1996; Tyler *et al*., 1998; Iguchi *et al.*, 2001).

Rainbow trout (Oncorhynchus mykiss)

Binding – endogenous receptors

22. A cytosol fraction prepared from rainbow trout (rbt) liver was used in a competitive binding assay for chemicals (Jobling *et al.*, 1995) (Table 1). Many of the compounds tested reduced the binding of the [^{3}H]-E2 to the rbtER. Butylbenzyl phthalate (BBP), di-*n*-butyl phthalate (DBP), di(2-ethylhexyl) phthalate (DEHP), di(2-ethylhexyl) adipate, benzophenone (BP), *n*-butylbenzene, 4-nitrotoluene, butylated hydroxyanisole, and 2,4-dichlorophenol reduced the binding of [^{3}H]-E2 to the receptor. Musk ketone, musk xylene, *p*-toluene, butylated hydroxytoluene, caffeine, cholesterol, *p*-hydroxybenzoic acid, *p*-tert butylbenzoic acid, 3,4-dimethylphenol, and 2-methylphenol did not impair binding of [^{3}H]-E2.

23. Relative binding affinities of endocrine active compounds to rbtER and fathead minnow (fhm) ER were studied (Denny *et al.*, 2005) (Table 1). Liver cytosol was prepared from individual trout liver or pooled fathead minnow livers (30-50 fish, separated by gender). Prior to use in competitive binding experiments, saturation binding with E2 was performed to determine the dissociation constant (K_d) and binding capacity (B_{max}) of each cytosolic preparation. The K_d values were higher for fhmER (2.6-22.3 nM) than for rbtER (0.8-2.7 nM); however, in both species, females and males had similar K_d (fhmER, 8.6 *vs.* 6.2 nM; rbtER, 1.7 *vs.* 1.2 nM). As with K_d, mean B_{max} values determined for fhmER preparations were higher for females (44.8 fmol/mg) than for males (24.8 fmol/mg). Mean B_{max} values for the rbtER were somewhat higher in females (17.7 fmol/mg) than males (12.5 fmol/mg). The mean half maximal inhibitory

concentration IC_{50} and Relative Binding Affinity (RBA) of 11 chemicals were determined with both fhmER and rbtER (4 C). The chemicals included E2, DES, EE2, estrone (E1), estriol (E3), tamoxifen (TAM), genistein (GEN), nonylphenol (NP), 4-*t*-octylphenol (OP), methoxychlor (MXC), T and methyltestosterone (MT). RBA was calculated for each chemical relative to E2 binding to the receptor. The estrogens DES, EE2 and E1 bound with high affinity to both receptors, with respective RBAs of 586, 166 and 28% (fhmER) and 179, 89 and 5% (rbtER). RBA of E3, TAM and GEN for both fhmER and rbtER were moderate, with values between 0.3 and 5%. The alkylphenols had weak affinity for the ERs with RBAs for the fhmER of 0.1 and 0.01 for NP and OP, respectively. Corresponding values for the rbtER were 0.027 and 0.009. [^{3}H] E2 was only partially displaced from both the fhmER and the rbtER by MXC, T and MT. Comparison of RBAs of the chemicals tested for fhmER and rbtER indicates that the rank order of RBAs essentially are the same for both species.

24. Rainbow trout ER from liver tissue competitive binding assays with 11chemicals, (E2, DES, 4-hydroxytamoxifen (4-HT), GEN, NP, *o,p'*-DDT, MXC, monohydroxyMXC, dihydroxyMXC, kaempferol and resorcinol sulphide) yielded calculable RBA from 179 to 0.0006% and no binding for 3 additional chemicals (4-*tert*-butylphenyl salicylate, 4,4'-butylidene bis(6-*tert*-butyl-*m*-cresol and brompheniramine hydrogen maleate). E2, DES, MXC, *o,p'*-DDT, MXC, dihydroxyMXC, kaempferol and resorcinol sulphide had produced complete displacement curves in binding assays, including the lowest affinity binder with an RBA of 0.0006%. Two chemicals with only partial binding curves up to their solubility limit did not induce VTG. (Schmieder *et al.,* 2004) (Table 1). Relative affinity of chlordecone and *o,p'*-DDT or DDE for rbtER was studied. Chlordecone had relatively low affinity (1000-fold less than moxestrol, a synthetic estradiol) for hepatic rbtER. *o,p'*-DDT and *o,p*'-DDE, but not *p,p*'-DDE, also exhibited low binding affinity (approximately 156000-fold less than moxestrol). These data indicated that chlordecone, *o,p*'-DDT and *o,p*'-DDE were weakly estrogenic in juvenile trout (Donohoe and Curtis, 1996) (Table 1). As is also suggested by the trend toward higher proportions of female medaka upon treatment of 1-day post-hatch with *o,p'*-DDT for 100 days (see Dietrich and Krieger (2009) for a review)

25. Representative alkylphenols such as phenol, pentylphenol, hexylphenol, heptylphenol, OP, NP, dodecylphenol, and phthalates such as di(2-ethylhexyl) phthalate (DEHP), diaryl phthalate, dinonyl phthalate, butylbenzyl phthalate (BBP), dibutyl phthalate (DBP) and diethyl phthaltate, the pesticides dieldrin and toxaphene, the mycoestrogen zearalenone (ZEA) and the phytoestrogen GEN were tested for their ability to displace endogenous ligand from putative rbtER, AR and cortisol receptor (CR) from rainbow trout liver and brain (Knudsen and Pottinger, 1999) (Table 1). The rbtER displayed a higher affinity for alkylphenols than for phthalates, but both groups of compounds were 10^4 – 2 x 10^5 times less potent than E2 in displacing specifically bound [^{3}H]-E2. Toxaphene and dieldrin did not bind to rbtER, either alone or in combination. ZEA and GEN were about 10^3-fold less potent than E2 and showed no increase in potency when tested in combination. None of the compounds tested showed evidence of binding to the rbtAR or the rbtCR. It is concluded that the compounds tested are exclusively, albeit weakly, estrogenic in rainbow trout and do not display any synergistic effects.

26. The estrogenicity of ZEA and its metabolites (α- and β-zearalenol) was evaluated using an *in vitro* competitive receptor binding assay (Arukwe *et al.*, 1999) (Table 1). The ER binding affinities of α-zearalenol and ZEA in rainbow trout were approximately 1/150 and 1/300 to that of E2, respectively. Generally, α-zearalenol and ZEA possess estrogenic potencies that are approximately 50% compared to that of E2, and their order of estrogenic potency *in vitro* receptor competitive binding is α-zearalenol > ZEA > β-zearalenol in this study (Arukwe *et al.,* 1999) (Table 1).

27. Rainbow trout ER from the livers of mixed sex fish exhibited [^{3}H]-E2 binding characteristics, and livers from female fish contained 2-3 times higher amounts of ER than livers from the males. In competition studies with [^{3}H]-E2, the rbtER were found to bind both native steroids (E2 > E1 > E2 17-glucuronide >> T and 11-ketotestosterone (11-KT)) and putative estrogen mimics (DES, 4-HT, EE2 >

GEN, ZEA > OP, NP, and *o,p'*-DDT. The pesticides toxaphene and dieldrin, which are proposed to bind to and activate hER, did not display significant binding affinity for the rbtER (Tollefsen *et al.*, 2002) (Table 1).

Binding – recombinant receptors

28. A study investigated the ability of 34 natural and synthetic chemicals to compete with [^{3}H]-E2 for binding to bacterially expressed glutathione *S*-transferase (GST)-ER fusion proteins from five different species (Matthews *et al.*, 2000) (Table 1). Fusion protein constructs consisted of the ER D, E and F domains of hERα (GST-hERαdef), mouse ERα (GST-mERαdef), chicken ER (GST-cERdef), green anole ER (GST-gaERdef) and rbtERs (GST-rbtERdef). All five fusion proteins displayed high affinity for E2 with *Kd* values ranging from 0.3 to 0.9 nM. Although, the fusion proteins exhibited similar binding preferences and binding affinities for many of the chemicals, several differences were observed. For example, α-zearalenol bound with greater affinity for GST-rbtERdef than E2, which was in contrast to other GST-ERdef fusion proteins examined. Coumestrol, GEN and naringenin bound with higher affinity for the GST-gaERdef, than to the other GST-ERdef fusion proteins. Many of the industrial chemicals examined preferentially bound to GST-rbtERdef. Bisphenol A (BPA), OP and *o,p'*-DDT bound with an approximately 10-fold greater affinity to GST-rbtERdef than to other GST-ERdefs. MXC, *p,p'*-DDT, *o,p'*-dichloro-diphenyl-dichloroethylene (DDE), *p,p'*-DDE, α-endosulfan and dieldrin weakly bound to the ERs from the human, mouse, chicken and green anole. In contrast, these compounds completely displaced [^{3}H]-E2 from GST-rbtERdef. These results demonstrate that ERs from different species exhibit differential ligand preferences and relative binding affinities for estrogenic compounds and that. These differences may be due to the variability in the amino acid sequence within their respective ER ligand binding domains, thus additional ER binding assays using fish ERs should be developed.

Transcriptional Activation assay – endogenous systems

29. Eleven lower to no affinity chemicals (RBA < 0.1%) were tested in trout liver slices to measure induction of rbtER-dependent VTG mRNA in the presence of chemical passive partitioning (from media to multiple hepatocyte layers in the slice) and liver xenobiotic metabolism. VTG induction in slices was observed in a concentration-dependent manner for 8 chemicals (E2, DES, MXC, *o,p'*-DDT, MXC, dihydroxyMXC, kaempferol, resorcinol sulphide) tested that had produced complete displacement curves in binding assays, including the lowest affinity binder with an RBA of 0.0006%. Two chemicals with only partial binding curves up to their solubility limit did not induce VTG. The monohydroxy metabolite of MXC was the only chemical tested that apparently bound rbtER but did not induce VTG mRNA(Schmieder *et al.*, 2004) (Table 1).

30. VTG induction response in isolated trout liver cells has been suggested as *in vitro* screening for identifying estrogen-active substances (Jobling and Sumpter, 1993; Kwon *et al.*, 1993; Pelissero *et al.*, 1993; Gagné and Blaise, 1998; Valliant *et al.*, 1998; Islinger *et al.*, 1999; Shilling and Williams, 2000; Okoumassoun *et al.*, 2002; Olsen *et al.*, 2005). The main advantages of the hepatocyte VTG assay are considered its ability to detect effects of estrogenic metabolites, since hepatocytes *in vitro* remain metabolically competent, and its ability to detect both estrogenic and anti-estrogenic effects (for reviews see, Navas and Segner, 2006; Iguchi *et al.*, 2006b).

Transcriptional Activation – engineered systems

31. An estrogen-responsive reporter gene assay was established using rainbow trout gonad cell line RTG-2 in which an acute estrogenic response is created by co-transfecting cultures with an expression vector containing rbtER cDNA in the presence of an estrogen-dependent reporter plasmid (Fent, 2001) (Table 2). RTG-2 cells were stably transfected with the rbtERα cDNA expression vector, and clones responsive to E2 were selected (Ackermann et al., 2002) (Table 2). The estrogenic activity of E2, EE2, NP, nonylphenoxy acetic acid, OP, BPA, *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE, *o*,p'-dichloro-diphenyl-dichloroethane (DDD), p,p'-DDD, and 2,2-bis(*p*-chlorophenyl)acetic acid (*p,p'*-DDA) was assessed at increasing concentrations. All compounds except *o,p'*-DDT, *p,p'*-DDE, and *p,p'*-DDA showed logistic dose-response curves, which allowed the calculation of lowest-observed-effect concentrations (LOEC) and the concentrations at which half-maximal reporter gene transcriptional activities were reached (EC_{50}). To check whether estrogen-responsive RTG-2 cells may be used to detect the estrogenic activity of environmental samples, an extract from a sewage treatment plant effluent was assessed and found to have estrogenic activity corresponding to the transcriptional activity elicited by 0.05 nM of E2. Dose-response curves of NP, OP, BPA, and *o,p'*-DDD revealed that the RTG-2 reporter gene assay is more sensitive for these compounds when compared to transfection systems recombinant for mammalian ERs.

32. A study of the potential for temperature to influence estrogen-mediated responses in poikilothermic animals (Sumida *et al.*, 2003) suggested that temperature may be an important variable to consider when using an estrogen-responsive reporter gene in a rainbow trout cell line to test the estrogenic activity of chemicals. Rainbow trout hepatoma cells (RTH 149) incubated at 11 or 18°C were co-transfected with an estrogen-responsive luciferase reporter plasmid and a plasmid containing a constitutively expressed rbtER. The RTH-149 cells were then exposed to E2, with samples collected at 24-h intervals (Hornung *et al.*, 2003) (Table 2). The 72-h EC_{50} for estrogen-responsive luciferase activity was 3.8×10^{-9} M at 11°C and 7.4×10^{-10} M at 18°C. The efficacy of E2 was lower at 11°C. The maximal response to E2 in cells at 11°C was 2.6-fold greater than controls, whereas the maximal response at 18°C was 3.2-fold greater than controls. EE2 was similar to E2 in potency (relative potency = 0.8) and efficacy at the two temperatures. The EC_{50} of the weak ER agonist 4-tert-pentylphenol was 7.6×10^{-7} M at 11°C and 6.9×10^{-7} M at 18°C; its potency relative to E2 was not significantly different at the two temperatures, 0.00036 and 0.00054 at 11°C and 18°C, respectively. The estrogen-responsive reporter gene activity produced by 10^{-8} M E2 was completely inhibited by the anti-estrogens ZM 189,154 and ICI 182,780, at 10^{-6} M concentration of either antagonist. Although there may be slight differences in responses between the two temperatures tested, this assay can be used to effectively determine the relative estrogenic activity of chemicals within the physiological temperature range of rainbow trout.

33. Cadmium (Cd)-mediated inhibition of vitellogenesis was studied in rainbow trout collected from contaminated areas or undergoing experimental exposure to Cd, and correlated with modification in the transcriptional activity of the ER. A recombinant yeast system expressing rbtER or hER was used to evaluate the direct effect of Cd exposure on ER transcriptional activity (Guevel *et al.*, 2000) (Table 1). In recombinant yeast, Cd reduced the E2-stimulated transcription of an estrogen-responsive reporter gene. *In vitro* binding assays indicated that Cd did not affect ligand binding to the receptor. Yeast one- and two-hybrid assays showed that E2-induced conformational changes and receptor dimerization were not affected by Cd. Conversely, DNA binding of the ER to its cognate element was dramatically reduced in gel retardation assay. This result suggests that Cd could be an important endocrine disrupter through a direct effect on ER transcriptional activity and may affect a number of estrogen signalling pathways.

Spotted sea trout (*Cynoscion nebulosus*)

Binding – endogenous receptors

34. Spotted sea trout (ss) ERs were identified in cytosolic (K_d =1.26) and nuclear (K_d =1.96) extracts of livers of adult female. The K_d did not differ between males and females or between vitellogenic and non-vitellogenic females. The binding in both the cytosolic and nuclear extracts was specific for estrogens (DES > E2 >> E1 = E3) (Smith and Thomas, 1990) (Table 1). ssER concentrations in cytosolic extracts from late vitellogenic females were significantly higher than those from non-vitellogenic females. The nuclear binding capacity of livers from mid-vitellogenic females (1.12 pmol/g liver) was significantly higher than the binding capacity in livers from non-vitellogenic females (0.16 pmol/g liver), but not that of late vitellogenic females (0.80 pmol/g liver). The concentration of E2-binding sites was greatest in the liver (liver >> ovary > heart> spleen > muscle > brain). No interference from other steroid-binding proteins was detected using a simple dextran-coated charcoal method to separate bound from free hormone. Approximately 14% of the binding in the cytosolic extract had DNA-binding affinity. ER binding activity was maximally extracted from nuclei with buffer containing 0.6 M potassium chloride. Nuclear receptors eluted from gel filtration columns with an apparent molecular weight of 95 kDa.

35. Several xenobiotics were tested for their ability to bind to the hepatic ssER (Thomas and Smith, 1993). Incubation of cytosolic extracts with the antiestrogens clomiphene, TAM, and nafoxidine caused displacement of [^{3}H]-E2 from ssER. Kepone (chlordecone) also bound to ssER, but it had a lower affinity than that observed with mammalian ER. These compounds have antiestrogenic or estrogenic actions in the spotted sea trout. However, several DDT derivatives and Poly-Chlorinated Bi-phenyls (PCB) mixtures failed to displace E2 from the ssER, even though they have been shown to bind to mammalian ER. The lack of binding to ssER by several of the xenobiotics may be due to structural differences between ER in spotted sea trout and in mammals. These results indicate that the ssER assay can be used for screening xenobiotics for possible estrogenic activity in teleosts.

Mummichog (Fundulus heteroclitus)

Binding – recombinant receptors

36. In *Fundulus heteroclitus*, ERα (fhERα) was cloned and the ERα binding assay was carried out (Urushitani *et al.,* 2003) (Table 1). Recombinant fhERα LBD fusion protein was prepared using *E. coli*. fhERα binding affinities to various concentrations ($1 \times 10^{-4} – 1 \times 10^{-11}$ M) of OP, NP, BP, di-*n*-buthylhexyl phthalate (DBHP), octachlorostyrene (OCS) and tributyltin chloride (TBT) were measured. E_2 bound to fhERα with an IC_{50} value of 5.5×10^{-9} M. The RBA values of the other chemicals were calculated as a percent ratio of the IC_{50} values of test substances relative to E2. RBA of OP and NP for fhERα were approximately 0.65 and 0.42% of E2, respectively. BP exhibited weak binding affinity, approximately 0.008% of E2. Phthalates also showed weak binding affinities (0.01 - 0.02% of E2). DBHP showed slightly higher receptor binding affinity ability for the fhERα. The binding potency of OCS to fhERα was estimated as 0.02% of E2.

Medaka (Oryzias latipes)

Binding – recombinant receptors

37. Medaka (m) ERα and mERβ binding assays were carried out using recombinant LBD fusion protein prepared using *E. coli* (Nakai, 2003) (Tables 1, 2 and 3). RBA of E2 was used for evaluation. RBA of OP and NP for mERα were 16 and 8.1% of E2, respectively. DEHP, buthylbenzyl phthalate and TBT exhibited RBAs of 0.79, 0.23 and 0.1%, respectively. DBP, OCS, dicyclohexyl phthalate, diethyl phthalate and di-2-ehylhexyl adipate showed weak binding affinities (0.012-0.045%). RBA of OP, NP and DEHP for mERβ were 0.8-0.83%. Phthalates showed weak binding affinities (0.002-0.006% of E2).

Transcriptional Activation – engineered systems

38. For reporter gene assay, HeLa cells were transiently co-transfected with both receptor and reporter plasmids in a serum-free medium (Nakai, 2003). Cells were exposed to chemicals (1×10^{-5} - 1×10^{-11} M), then firefly luciferase luminescence was detected. Relative potencies of OP and NP to E_2 for mERα were 1.3 and 0.35%, respectively. Other chemicals including phthalates and TBT were negative. For mERβ and mAR reporter gene assay (value obtained for 5α-dihydrotestosterone (DHT) was 100%), all the chemicals studied, including phthalates, alkylphenols, TBT and OCS were negative (Table 3).

Transgenic animal system

39. The detection of environmental estrogens using *in vivo* tests offers several advantages when compared to the computational chemical analysis performed on a compound by compound basis. These advantages include the screening of novel contaminants with biologically meaningful endpoints, the integration of absorption, distribution, metabolism, and excretion (ADME) processes. In this holistic sense, the evaluation of potential toxic effects due to endocrine disrupting chemicals requires validated *in vivo* 'bio-tests'. Critical disadvantages of whole organism bio-tests are they are laborious and time-consuming, have periodic low sensitivity and raise animal welfare concerns. To overcome these problems, a transgenic medaka (ChgH-GFP) strain harbouring the green fluorescence protein (GFP) gene driven by choriogenin H (ChgH) gene regulatory elements was developed (Kurauchi *et al.*, 2005). ChgH is an egg envelope protein induced by estrogens in the liver. With yolk sac larvae of this strain, GFP induction in liver was observed 24 h after onset of aqueous exposure to 0.63 nM E2, 0.34 nM EE2, or 14.8 nM E1. Concentrated sewage treatment effluent induced GFP expression. Comparison of E2 equivalents estimated by GFP-induction in transgenic medaka, a Yeast Estrogen System (YES) assay, and Gas Chromatography-Mass Spectrometry (GC/MS) showed detection limits in the same order of magnitude. These results indicated that the sensitivity of the transgenic medaka strain is sufficient for application as an alternative model in monitoring environmental water samples for estrogenic chemicals.

40. A method for quantification of GFP in ChgH-GFP strain of medaka using image analysis was established and applied for the analysis of time- and concentration-dependent GFP fluorescence in juvenile fish (Scholz *et al.*, 2005). Concentration-response analyses were performed with fish exposed for 14 days to E2 (0.37-367 pM), GEN (0.37-367 nM), or NP (0.367-1,835 nM). By means of image analysis, it was shown that ChgH-GFP was induced by E2 at 183.5 pM or higher concentrations. Time-course and recovery experiments indicated a strong accumulation of GFP in the liver. Results of RT-PCR analysis of ChgH and VTG demonstrated induction of gene expression for the same range of concentrations as that for GFP

analysis. Neither expression of these genes nor GFP fluorescence was induced by GEN and NP. Although the ChgH-GFP strain failed to detect these weakly estrogenic compounds, the simplicity of the GFP quantification during early life stages of fish offers promising possibilities for further developments of transgenic strains using different target regulatory sequences.

Fathead minnow (Pimphales promelas)

Binding – endogenous receptors

41. Relative binding affinities of endocrine active compounds to rbtER and fhmER were studied (Denny *et al.,* 2005, study summarized in paragraph 18). Hornung et al. (2004) reported competitive binding of MT to the fhmER with a RBA of 68.3% of E2 as part of a study that demonstrated MT can be aromatized to MT via aromatase activity. Rider *et al.* (2009a) showed that E2 displayed a reduction in binding to fhmERα at an elevated temperature (37ºC) compared to 23ºC.

Zebrafish (*Danio rerio*)

Transcriptional Activation – engineered systems

42. Three functional ER forms have been cloned and characterized in zebrafish (zf), zfERα, zfERβ1 and zfERβ2 (Menuet *et al.,* 2002) (Table 2). Identity was 40.5% between zfERα and zfERβ2, and 51.5% between zfERβ1 and zfERβ2, and the overall amino acid sequences for these receptors were different. The percentages of identity between these receptors suggest the existence of three distinct genes. Each cDNA encoded a protein that specifically bound E2 with dissociation constant 0.4 nM for zfERβ2 and 0.75 nM for both zfERα and zfERβ1, indicating that zfERβ2 has a 1.8-fold higher affinity for E2 than do the two other forms. In transiently co-transfected Chinese hamster ovary (CHO) cells with zfER expression vectors and a luciferase reporter gene, ERE-TK-Luc, all three forms were able to induce the expression of the reporter gene driven by a consensus ERE in a dose-dependent manner, from 5×10^{-11} M E2. The zfERβ2 was slightly more sensitive than zfERα and zfERβ1. ICI164384 or 4-HT did not activate zfERs, and 100-fold excess of these compounds completely suppressed E2 stimulation of the reporter gene mediated by each zfER. All three zfERs were activated by DES, E1 and E3. In contrast, T and progesterone (P) were unable to induce expression of the reporter gene. Tissue distribution pattern, analyzed by RT-PCR, showed that the three zfER mRNAs largely overlap and are predominantly expressed in brain, pituitary gland, liver and gonads. *In situ* hybridization showed that zfER mRNAs exhibit distinct but partially overlapping patterns of expression in preoptic area and the mediobasal hypothalamus in the brain.

43. The polycyclic musks 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) and 1,2,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-gamma-2-benzopyran (HHCB) have been used as fragrance ingredients in perfumes, soaps and household cleaning products. They are known to be ubiquitously present in the aquatic environment and, because of their lipophilic nature, tend to bioaccumulate in aquatic biota. In surface waters, concentrations between 1 ng/L and 5 μg/L were found, depending mainly on the proportion of sewage effluents in the water. In fish, under normal environmental conditions, concentrations in the microgram per kilogram fresh weight range were found. AHTN and HHCB exerted mainly anti-estrogenic effects on the hERα and hERβ in an *in vitro* reporter gene assay (Schreurs *et al.,* 2002). The *in vitro* anti-estrogenic effects of both musks were assessed on zebrafish ERs (Legler *et al.,* 2002). Antagonism was observed on zfERβ and was more pronounced on zfERγ.

44. Functional *in vitro* reporter gene assays were developed for the rapid determination of exposure to (xeno)estrogens. The *in vitro* ER-mediated Chemically Activated LUuciferase gene eXpression (ER-CALUX) assay used T47D human breast cancer cells stably transfected with an ER-mediated luciferase gene construct (Murk et al., 1996). Luciferase reporter gene activity can be easily quantified following short-term exposure to chemicals activating endogenous ERs (Legler et al., 2002) (Table 2).In the *in vitro* ER-CALUX assay, EE2 and E2 were equipotent, although the xenoestrogens *o,p'*-DDT and NP were full estrogen agonists. Using transiently transfected recombinant ER and reporter gene constructs, EE2 also showed relatively potent activation of zfERα and zfERβ compared to hERα and hERβ. The zfERβ and zfERγ showed higher transactivation by (xeno)estrogens relative to E2 than hERβ.

Transgenic animal system

45. In the *in vivo* assay, transgenic zebrafish were used in which the same luciferase construct had been stably introduced (Murk *et al.,* 1996). Luciferase reporter gene activity can be easily quantified following short-term exposure to chemicals activating endogenous ERs. Exposure to E2, E1, EE2, *o,p'*-DDT, NP, and DEHP revealed that EE2 was the most potent (xeno)estrogen tested and was 100 times more potent than E2 in the transgenic zebrafish assay (Legler et al., 2002) (Table 2). *o,p'*-DDT demonstrated weak dose-related estrogenic activity *in vivo*.

46. Using a transgenic zebrafish assay, antiestrogenicity of the musks (AHTN and HHCB) was studied *in vivo*. Dose-dependent antagonistic effects were observed at concentrations of 0.1 and 1 µM AHTN and HHCB. GC-MS analysis showed that the musks bioaccumulated in the fish, with internal concentrations (15-150 mg/kg fresh weight) which were roughly 600 times higher than the nominal test doses. These results indicate that environmental contaminants have anti-estrogenic activity in an *in vivo* fish assay that focuses solely on ER-mediated effects (Schreurus *et al.,* 2004).

Channel catfish (Ictalurus punctatus)

Binding – endogenous

47. Estrogen Receptors from channel catfish (ccER) hepatic tissue was characterized for binding affinity of several compounds. Affinity was indirectly measured as potency of the chemical for inhibiting binding of radiolabeled E2 to specific binding sites (Nimrod and Benson, 1997) (Table 1). The order of potency among therapeutic chemicals was EE2 > unlabeled E2 = DES > mestranol > TAM >> T. Unlabeled E2 had an IC_{50} of 2.2 nM. Several environmentally relevant chemicals were evaluated in a similar manner and the order of potency established was the *O*-demethylated metabolite of MXC > NP > chlordecone > MXC > *o,p'*-DDT > *o,p'*-DDE > β-hexachlorocyclohexane. Demethylated MXC had an IC_{50} 1000-fold greater than that of E2. Of the most potent inhibitors, NP appeared to be a competitive inhibitor for the same binding site as E2, while *O*-demethylated MXC had a more complex interaction with the receptor protein. ER from non-vitellogenic females was determined to have a *Kd* value of 1.0 to 1.3 nM. Because E2 has been reported to up-regulate teleostean ER, the hepatic ER population following *in vivo* xenobiotic exposure was assessed. NP significantly increased ER per milligram hepatic protein almost to the same extent as E2, but did not increase *Kd* to the same extent as E2.

Binding – recombinant receptors

48. Recombinant ERα and ERβ were obtained after transient transfection of COS7 cells with ER expression plasmids. NP and OP displayed some ability to displace [^{3}H] E2 from ERα and ERβ at high

concentrations, but dieldrin and atrazine had little binding activity for both ER subtypes and endosulfan for ERβ. The xenobiotics tested generally showed equivalent or greater affinity for ERα than ERβ, whereas endogenous estrogens, such as E2, E1 and E3, had much greater affinity for ERβ than ERα. These observations suggest that results of studies using fish ER extracts should be interpreted with caution, since both ER subtypes may be present (Gale *et al.*, 2004) (Table 1).

Atlantic croaker (Micropogonias undulatus)

Binding – endogenous receptors

49. Atlantic croaker (at) ER was identified in cytosolic and nuclear fractions of the testis as an example of marine teleost. A single class of high affinity, low capacity, and displaceable binding sites (atER) was identified by saturation analysis (*Kd*=0.40 nM in cytosolic extracts and *Kd*=0.33 nM in nuclear extracts). Competition studies demonstrated that the receptor was highly specific for estrogens (DES > E2 >> E1 = E3) and also bound several anti-estrogens. T and dihydrotestosterone (DHT) had much lower affinities for atER, whereas no displacement of specific binding occurred with 11-KT or any of the C21 maturation-inducing steroids. A variety of xenoestrogens, including DDT, chlordecone (Kepone), NP, hydroxylated polychlorinated biphenyls (PCBs), and ZEA, bound to atER with relatively low binding affinities, 10^{-3} to 10^{-5} that of E2. A comparison of the binding affinities of various ligands for the testicular atER and the hepatic atER revealed that the testicular atER was saturated at a lower [^{3}H]-E2 concentration (1 nM *vs.* 4 nM). The binding affinities of several compounds, including T and nafoxidine, exhibited marked differences for the two ERs; and most of the estrogens and xenoestrogens tested had higher binding affinities for the testicular receptor (Loomis and Thomas, 1999) (Table 1). Minor amounts of E2 (0.12 ng/g tissue/h) were produced by testicular tissue fragments incubated *in vitro*, and E2 was detected in male Atlantic croaker plasma. The identification of a testicular ER and evidence that E2 is produced by the testes in Atlantic croaker suggest that estrogens participate in the hormonal control of testicular function in teleosts.

Atlantic salmon (*Salmo salar*)

Binding – endogenous receptors

50. Atlantic salmon hepatic ER (asER) was partly characterized, and the ligand-binding preference for a range of endogenous steroids and environmental estrogen mimics was determined by receptor-radio ligand studies (Tollefsen *et al.,* 2002) (Table 1). The results show that Atlantic salmon livers contain ERs that bind [^{3}H]-E2 with high affinity and low capacity (*Kd* = 2.5-4.4 nM and B_{max} = 27-97 fmol/mg protein). Atlantic salmon ER exhibit similar [^{3}H]-E2 binding characteristics, although livers from female fish contained a 2-3 times higher amounts of ER than the males. In competition studies with [^{3}H]-E2, the asER was found to bind both native steroids (E2 > E1 > E2 17-glucuronide >> T and 11-KT) and putative estrogen mimics (DES, 4-HT, EE2 > GEN, ZEA > OP, NP, and *o,p'*-DDT). The pesticides toxaphene and dieldrin, which are proposed to bind to and activate hER, did not display significant binding affinity for the fish ER, however, in general, the asER was found to bind both native steroids and estrogen mimics with similar affinity and specificity. The present results suggest that closely related species such as Atlantic salmon and rainbow trout display similar ER ligand-binding requirements, although interspecies differences in ER affinity and specificity between divergent species such as fish and humans may exist.

Common carp (Cyprinus carpio)

Binding - endogenous receptors

51. Competitive receptor binding assays using common carp hepatic ER were conducted. The results indicated the presence of a single class of estrogen binding sites with high affinity and limited capacity in liver cytosol of carp. The various test agents showed partly quantitative differences in their binding affinities, with the xenobiotics generally showing limited ability to displace [^{3}H]-E2 from the hepatic ER of carp. The affinity ranking for ligands on the basis of statistical differences of the IC_{50} values were E2 > TAM > BPA > OP > 3,3,4',4-tetrachlorobiphenyl > diethylphtalate > NP > butylhydroxyanisol > p,p'-DDT > Arochlor 1254, MXC, 3-methylchlanthrene, β-naphtoflavone, anthracene and prochloraz (Kloas *et al.*, 2000) (Table 1).

Red sea bream (*Pagrus major*)

Binding – recombinant receptors

52. Recombinant red sea bream ERα ligand binding domain was expressed in *E. coli* and the binding affinities of OP and NP were examined (Nakai *et al.,* 2003) (Table 1). Scatchard analysis revealed a best fit for a one-site model with a *Kd* value of 3.01 nM. The relative binding affinities of OP and NP to the receptor were 1.7% and 0.97% of E2, respectively.

Roach (Rutilus rutilus)

Transcriptional Activation – engineered systems

53. The full-length cDNAs for ERα (1680 bp) and ERβ (1812 bp) were cloned from wild male roach (*Rutilus rutilus*) living in rivers in the United Kingdom and characterized in the roach and their patterns of expression established in the body/gonad and head/brain during early life through the period of gonadal sexual differentiation (Katsu *et al.*, 2007a) (Table 2). Transactivation assays were developed for both roach ER subtypes and the estrogenic potencies of steroidal estrogens differed markedly at the different ER subtypes. EE2 was by far the most potent chemical and E1 (the most prevalent environmental steroid in wastewater discharges) was equipotent with E2 in activating the ERs. Comparison of the EC_{50} values for the compounds tested showed that ERβ was 3-21-fold more sensitive to natural steroidal estrogens and 54-fold more sensitive to EE2 as compared to ERα. These findings add substantial support to the hypothesis that steroidal estrogens play a significant role in the induction of intersex in roach populations in rivers in the United Kingdom and that the molecular approach described could be usefully applied to understand interspecies sensitivity to xenoestrogens.

Mosquitofish (Gambusia affinis affinis)

Transcriptional Activation – engineered systems

54. Full-length mosquitofish ER (mqfER) cDNAs were obtained using cDNA library screening and Rapid Amplification of cDNA (RACE) techniques. Amino acid sequences of mqfERs showed over-all homology of 46% (α *vs.* β1), 43% (α *vs.* β2), and 52% (β1 *vs.* β2). ERE-luciferase reporter assay system was applied to characterize these receptors. In this transient transfection assay system using mammalian cells, the mqfER proteins displayed estrogen-dependent activation of transcription (Katsu *et al.*, 2007b) (Table 2).

Species differences

55. Using *in vitro* competitive enzyme immunoassay for ERs using ER-LBD proteins from human, *Xenopus laevis*, Japanese quail and Japanese medaka, the species specificity of the ability of NP and OP to bind ERs was analyzed. Although a significant difference was not detected among ERβ of human, quail and medaka, NP and OP exhibited the higher affinity for the mERα than hERα. These results indicate the species specificity of the capacity of chemicals to bind ERs (Nishizuka *et al.*, 2004). The RBA of OP and NP for fhmERα was higher, than that of hERα (Urushitani *et al.*, 2003).

56. Sumida *et al.*, (2003) (Table 2) reported on the analysis of species differences in ER-dependent transactivation with some chemicals using reporter gene assays. Full-length ER cDNAs from human, rat, chicken, caiman, whiptail lizard, *X. laevis* and rainbow trout were prepared from hepatic mRNA by the RT-PCR method and inserted into expression plasmids. Both expression and reporter plasmids were transiently transfected into HeLa cells, and then the estrogenic effects of chemicals such as E2, BPA, GEN, NP, DES, daidzein, *o,p'*-DDT, dieldrin, 4-HT, and raloxifene, were analyzed in terms of induction of luciferase activity. For agonist assays, when 1 or 10 pM E2 was added, HeLa cells exhibited an increase in luciferase activity with human, rat, chicken, caiman, whiptail lizard and *X. laevis*. In the case of rainbow trout, they showed an increase in luciferase activity when 1 or 10 nM E2 was added. Rainbow trout ER demonstrated a significant dose-response shift, requiring around a 100-fold higher E2 concentration than the hER in HeLa cells. For antagonist assays, 4-HT and raloxifene caused a decrease in luciferase activity when it was added with E2. With rbtER, increase of luciferase activity was observed at concentrations of 10-100 nM E2. No species differences in transactivation were found among human, rat, chicken, alligator, whiptail lizard and *X. laevis* ERs. Luciferase assay using E2 were also carried out with BF-2 cells from the bluegill fry, incubated at 24°C. The results using BF-2 cells were similar those using HeLa cells. The rbtER showed a dose-response shift, requiring around 10-fold higher E2 concentration compared with hER in BF-2 cells. Thermo-dependent alteration in susceptibility to E2 was observed with the rbtER because of thermo-dependence of estrogen binding.

57. Strain differences in sensitivity of estrogenic chemicals (Iguchi *et al.*, 1987; Spearow *et al.*, 1999; Long *et al.*, 2000) and dioxin (Jena *et al.*, 1998; Karchner *et al.*, 2006; Kawakami *et al.*, 2006) have been reported in mice and rats. However, strain differences in sensitivity to chemicals have not been extensively studied in fish. Some information is available on sensitivity of species showing higher sensitivity to estrogenic chemicals in medaka than those of fathead minnow and zebrafish and vice versa (see Dietrich and Krieger (2009) for review), and equal sensitivity to androgenic chemicals (Seki *et al.*, 2006). Other study showed that zebrafish is a more sensitive species than medaka for 21-day VTG assay (Örn *et al.*, 2006). Albeit, depending on the endpoint used, medaka appear to be less sensitive to estrogenic and androgenic chemicals than other laboratory species (fathead minnow, Zebrafish, etc; see Dietrich and Krieger (2009); FIFRA-SAP (2009) for reviews).

IV. ANDROGEN RECEPTOR

58. Androgens are essential for the morphological specification of male type sexual characters that have evolved in each species presumably for survival and/or reproduction. Understanding the mechanisms of androgen-dependent organogenesis underlying the reproductive diversity among species is one of the central problems in evolutional biology. The androgen receptor (AR) belongs to the nuclear receptor (NR) super family and is the key molecule controlling the expression of such masculine phenotypes. In teleost fishes, two distinct paralogous copies of *AR*s have been identified from several species including Nile tilapia (*Oreochromis niloticus*), Japanese eel (*Anguilla japonica*), and Atlantic croaker (*Micropogonias undulatus*) (Ikeuchi *et al*., 1999: Todo *et al*., 1999; Sperry and Thomas, 1999). In rainbow trout (*Oncorhynchus mykiss*), two isoforms of *AR*, probably derived from salmonid tetraploidy, were cloned (Takeo and Yamashita, 1999). Male secondary sexual characters appear as an elongation of the fin ray, kidney hypertrophy, thickened skin, appearance of breeding colors and transition of anal fin to copulatory organ (Gonopodium) in teleost fishes (Borg, 1994; Ogino *et al.*, 2004; Sone *et al.*, 2005). Thus, AR gene duplication might contribute to the evolutionary divergence of secondary sexual characters in teleost fishes (Ogino *et al.,* 2009). It has been known that the ligand selectivity of AR is different among species (Leihy *et al.*, 2004). In mammals, T and DHT are considered to be effective ligands for AR (Quigley *et al.*, 1995). 11-KT is known as a potent androgen in teleost fishes (Borg, 1994). Development of the screening assay systems of chemicals showing androgenic acitivity and anti-androgenic activities are delayed as compared to ER systems, since AR gene sequences are larger than those of ER and androgen mimicking chemicals have not been studied in detail so far

Rainbow trout

Binding – recombinant receptors

59. Two AR cDNA clones (rbtARα and rbtARβ) from the rainbow trout testis were isolated (Takeo and Yamashita, 1999) (Tables 1 and 2). To investigate the functions of the rbtARα, the ligand binding ability of rbtARα were analyzed (Takeo and Yamashita, 2000). In ligand-competition experiments, T (IC_{50}: 3 x 10^{-9} M) competed with [^{3}H]-mibolerone binding for rbtARα slightly more potently than the teleost fish-specific natural androgen 11-KT (IC: 8 x 10^{-9} M), which is thought to be the functional spermatogenesis inducer. In contrast, T (EC_{50}: 5 x 10^{-9} M) and 11-KT (EC_{50}: 6 x 10^{-9} M) showed similar efficiency upon co-transfection into *epithelioma papulosum cyprini* (EPC) cells with an rbtARα expression vector and an androgen-responsive element-based reporter gene. These results indicate that activation of rbtARα does not distinguish between 11-KT and T and suggest that a specific system, which is mediated only by 11-KT, may exist in the rainbow trout.

60. Wilson et al. (2007) compared competitive binding of a set of compounds (R1881, MT, trenbolone, DHT, 11-KT, P, androstenedione, T, E2, M2, M1, hydroxyflutamide, viclozolin, flutamide, linuron, *p,p'*-DDE, ketoconazole, DBP, DEHP and atrazine) to full-length recombinant rbtAR, fhm AR and hAR, each expressed in COS cells. Saturation binding and subsequent Scatchard analysis using [^{3}H]R1881, a high-affinity synthetic androgen, revealed an equilibrium dissociation constant (*K*d) of 0.11 nM for the rbtAR, 1.8 nM for the fhmAR, and 0.84 nM for the hAR. Compounds, including endogenous and synthetic steroids, known mammalian anti-androgens, and environmental compounds, were tested for competitive binding to each of the three receptors. Overall, agreement existed across receptors as to binding versus non-binding (DEHP and atrazine) for all compounds tested in this study. Minor differences, however, were

found in the relative order of binding of the compounds to the individual receptors.

Fathead minnow

Binding – recombinant receptors

61. Both AR and ERα from fathead minnow were isolated and sequenced (Wilson *et al.,* 2004b) (Table 1). The fhmAR was expressed and characterized with respect to function using saturation and competitive binding assays in COS monkey kidney cells. Saturation experiments along with subsequent Scatchard analysis determined that the *Kd* of the fhmAR for the potent synthetic androgen R1881 was 1.8 nM, which is comparable to that for the hAR in the same assay system. In COS whole cell competitive binding assays, potent androgens such as DHT and 11-KT were also shown to be high affinity ligands for the fhmAR. Affinity of fhmAR was reported for a number of environmental contaminants including the AR agonists androstenedione and 17α- and 17β-trenbolone (TB); AR antagonists such as *p,p'*-DDE, linuron, and vinclozolin; and E2.

62. Effects of flutamide on endocrine function in the fathead minnow were characterized (Ankley *et al.,* 2004) (Table 1). Binding assays with whole cells transiently transfected with cloned fhmAR indicated that flutamide bound competitively to the receptor. However, as is true in mammalian systems, a 2-hydroxylated metabolite of flutamide binds to the AR with a much higher affinity than the parent chemical. Mixture experiments with flutamide and TB, a pharmaceutical, androgenic, anabolic steroid, demonstrated that the anti-androgen effectively blocked TB-induced masculinisation (nuptial tubercle production) of female fathead minnows, indicating antagonism of an AR receptor-mediated response *in vivo*. Conversely, reductions in VTG in TB-exposed females were not blocked by flutamide, suggesting that the VTG response is not directly mediated through the AR.

Mosquitofish (Gambusia affinis holbrooki/affinis)

Binding – recombinant receptors

63. Female mosquitofish (*Gambusia affinis holbrooki*) downstream from the Kraft paper mills in Florida display masculinisation of the anal fin, an androgen-dependent trait. Androgenic activity was determined in pulp-mill effluent (PME) from the Fenholloway River in Florida *in vitro* and this activity was related to the reproductive status of female mosquitofish taken from this river. . Eighty percent of the female mosquitofish from the Fenholloway River were partially masculinised while another 10% were completely masculinised, based upon the numbers of segments in the longest anal fin ray (18.0 *vs*. 28.1, p-value < 0.001, in the Econfina River *vs*. the Fenholloway River, respectively) (Parks *et al.,* 2001). In a COS whole cell-binding assay, all 3 PME samples displayed affinity for hAR (p-value < 0.001). In addition, PME induced androgen-dependent gene expression in CV-1 cells (co-transfected with pCMV hAR and Mouse Mammary Tumor Virus (MMTV) luciferase reporter), which was inhibited by about 50% by co-administration of hydroxyflutamide (1 µM), an AR antagonist. Water samples collected upstream of the Kraft mill or from the Econfina River did not bind hAR or induce luciferase expression. When CV-1 cells were transfected with human glucocorticoid receptor (hGR) rather than hAR, PME failed to significantly induce MMTV-luciferase expression. Further evidence of the androgenicity was observed using a COS cell AR nuclear-translocalization assay. PME bound hAR and induced translocalization of AR into the nucleus. In contrast, AR remained perinuclear when treated with water from the control sites (indicating the absence of an AR ligand). PME also displayed "T-like" immunoreactivity in a T radioimmunoassay, whereas water from the reference sites did not. Water collected downstream of the Kraft mill on the Fenholloway River contains unidentified androgenic substances whose presence is associated with masculinisation of female mosquitofish.

Transcriptional Activation – engineered systems

64. TB is a potent agonist of androgen receptors, and has been extensively used as a growth promoter for beef cattle in the US. The effects of TB on adult and newborn mosquitofish (*Gambusia affinis affinis*) and two forms of mosquitofish AR, mqfARα and mqfARβ, were reported (Sone *et al.*, 2005). TB induced differentiation of the anal fin into a gonopodium in fry of both sexes, stimulated precocious spermatogenesis in the testes of males and the formation of ovotestes in females. The transactivation of mosquitofish ARs (mqfARs) were examined using an androgen-responsive MMTV-luciferase assay system. Mosquitofish ARs showed androgen-dependent activation of transcription from the MMTV promoter (Katsu *et al.*, 2007b).

V. DISCUSSION

65. Extensive studies show that chemicals in the aquatic environment possess hormonal activities possibly influencing wildlife populations of aquatic organisms (Routledge *et al.*, 1998; Tyler *et al.*, 1998; Koplin *et al.*, 2002). Chemicals having these activities may reach the aquatic environment as components of municipal and industrial sewage outfalls and agricultural drainage. In the aquatic environment, estrogenic activity has primarily been ascribed to the natural steroids, E2, E1, E3, and a synthetic estrogen EE2, and to a lesser extent, NP, OP and BPA (Sumpter and Jobling, 1995). A wide variety of *in vitro* assays have been developed, such as receptor binding, cell proliferation, and gene activation (using endogenous receptors and/or gene products, or recombined receptor and reporter gene systems), that can be used to screen for hormonal activities and potency for prioritizing chemicals for further testing and for direct application for environmental monitoring. While most *in vitro* assays were initially developed using human receptor (hER and hAR), many systems are now available that use receptors from a variety of wildlife species as shown in this DRP. Each assay measures different end points at different levels of biological complexity of hormone action. All assays have their advantages and limitations. No single *in vitro* assay can on its own be regarded as ideal for assessing the hormonal and anti-hormonal activities by chemicals.

66. The liver cells of different fish species are especially well suited for use as a screen for xeno-estrogens and anti-estrogens that bind to the ER. The presence of ER in the liver, which is responsive to E2 stimulation resulting in the gene product VTG, that is easily measured as mRNA or protein, bypasses the need for insertion of receptors or reporter genes. The male fish liver maintains the functional ER-driven VTG response despite the fact it is not normally used. One of the main advantages of this system is that liver cells in culture maintain the endogenous receptor, activation factors and complete gene response systems so that any receptor cross-talk affecting the function of the ER and inducing (anti)-estrogenic processes can be detected. One of the main advantages of using liver cells is their maintained complete xenobiotic metabolizing capability enabling detection of effects of estrogen metabolites. Liver cells are also able to detect both estrogen and anti-estrogen effects. *In vitro* assay including primary hepatocyte cultures and liver slice assays have been developed based on these systems in fish and applied to chemical screening (*e.g.*, Shilling and Williams, 2000; Schmieder *et al.*, 2000, 2004; Navas and Segner, 2006). The systems allow detection of chemically-induced ER agonism or antagonism by measuring VTG mRNA or protein by a variety of techniques (Navas and Segner, 2006, 2008).

67. In hER, Kinnberg (2003) compared sensitivities among the binding assay, the cell proliferation assay using MCF-7 mammary tumour cells (E-screen), and the TA assays using T47D and MVLN cells, chimeric receptor in MCF-7, HeLa and HGELN cells, and the YES screen. Although the ER binding assay is fast, it is significantly less sensitive than the other *in vitro* assays. Cell cultures have some disadvantages associated with maintaining the cell line and avoiding contamination. On the other hand, their use in test assays offers significant advantages regarding sensitivity. Both reporter gene assays and the E-screen have been successfully applied to assess estrogenic activity in surface water and wastewater in numerous countries. Mammalian cells are more difficult and expensive to maintain in culture and are more susceptible to cytotoxic effects than yeast-based YES assays. Based on these evaluations, the YES-assay has been recommended to use for monitoring estrogenic activity in the contaminated environmental samples such as influent sewage containing toxic compounds (Kinnberg, 2003). One of the main concerns with YES-assays is the potential inability of some substances to cross the cell wall of yeast cells that could cross the cell membrane of vertebrate cells, and their lower sensitivity compared to other ER-TA assays at detecting estrogens in effluents (Folmar *et al.*, 2002). Also the YES assay is not sensitive for anti-estrogenic chemicals (Fang *et al.*, 2000). As for fish ER *in vitro* assays, some promising assays have been developed as shown above, there is not enough information available regarding the comparisons of

sensitivities among various assay systems. Therefore, sensitive and cheap *in vitro* assays systems using fish receptors needs to be evaluated, and if possible developed, validated and turned into OECD Test Guidelines.

68. It is difficult to compare sensitivities of *in vitro* hER assays with *in vivo* zfER assays since zfER has 3 isoforms and in the *in vivo* assays all the receptor and metabolic pathways are active. Estrogenic potency in wastewater treatment plant effluents has likewise been shown to be higher for *in vivo* studies than for *in vitro*. This may be due to the presence of specific estrogens, such as EE2, which are more potent estrogens in fish than in *in vitro* assays (Huggett *et al*., 2003). EE2 is the most potent estrogen largely due to its limited metabolization and thus longer systemic half-life. A 7-year, whole lake experiment at the Experimental Lakes Area in north-western Ontario, Canada, showed that chronic exposure of fathead minnow to low concentrations (5–6 ng/L) of EE2 led to production of VTG mRNA and protein, feminization of males impacting gonadal development as evidenced by intersex in males and altered oogenesis in females, and, ultimately, a near extinction of this species from the lake (Kidd *et al.*, 2007). This observation demonstrates that the concentrations of estrogens and compounds with similar estrogenic effects observed in freshwaters can impact the sustainability of wild fish populations and that EE2 can be used as a positive control for environmental estrogens.

69. Species comparisons in ER affinity have been presented by several investigators indicating that differences may be present in wildlife (Tollefsen *et al.*, 2002; Sumida *et al.*, 2003; Urushitani *et al.*, 2003; Nishizuka *et al.*, 2004; Denny *et al.*, 2005). However, care should be taken to ensure that differences noted are due to differences in the species and not due to the assays applied. For instance, chemical bioavailability and capacity for xenobiotic metabolization are two factors which can vary greatly across assay systems. Heringa *et al.* (2004) demonstrated methods for determining the free fraction in *in vitro* assays as a means of deriving assay independent EC_{50} values. Metabolic alteration of xenobiotics can alter both chemical form and availability has been demonstrated in some of the endogenously derived fish *in vitro* assay systems (Navas and Segner, 2006; Schmieder *et al.*, 2004). Additional information is needed on other assay systems to be able to differentiate between individual assay differences from species differences.

70. Screening and testing of chemicals binding to ERs emerge as an important issue in several regulatory programs or frameworks. Discrepancies exist, however, as to whether fish in vitro ERs assays should be included in regulatory testing and risk assessment. In view of the differences in binding affinities to ERα and ERβ and the significant contribution of ERβ to biological effects of chemicals, it remains unknown whether both types of ERs are needed for regulatory purposes. Recently, Dang (2010) collected publications on binding affinities of both mammalian and fish ERs for 65 chemicals, covering a wide range of strong, moderate, weak and non-ER binders. Systematic evaluation of the data was performed in order to compare the difference in binding affinity of chemicals to fish and mammalian ERs and to subtypes of ERs. Except for the reference estrogen E2, all 64 chemicals evaluated had different values of RBA, which resulted mostly from the inter-laboratory testing rather than due to inter-species differences. The author concluded that ER binding in one vertebrate species or one subtype of ERs could be extrapolated to other species or subtypes of ERs for most chemicals for regulatory purposes. Fish ERs are likely to be more sensitive to some weakly binding chemicals than mammalian ERs, suggesting the importance of including fish ERs in regulatory testing and decision making.

VI. CONCLUSIONS AND RECOMMENDATIONS

71. This draft DRP has focused on describing available test methods for estrogen and androgen fish receptor and TA testing, and on initial investigations of other types of assays with elements of fish receptors present, in addition to evaluating the usability of primary hepatocyte cultures and *e.g.* yeast-based assays with fish receptors. The purpose of this activity has been to compare assays and identify promising test methods that could be further developed, validated and hopefully turned into OECD Test Guidelines for regulatory purposes. From the present draft DRP it is evident that there are currently no assays that are validated and ready for being developed into OECD Test Guidelines, nor are there specific assays identified as being ready for validation at this stage. However, the draft DRP concludes that there are a number of promising areas, *i.e.* primary fish hepatocyte or yeast-based assays but further comparisons between assays in addition to inter-species comparisons are recommended areas for further work.

Primary Fish Hepatocyte Assays

72. Cultured primary fish liver cell assays are especially well suited for further considerations and potential developments for the following reasons: (i) The presence of ER in the liver, which is responsive to E2 stimulation resulting in the gene product VTG, that is easily measured as mRNA or protein, bypasses the need for insertion of receptors or reporter genes; (ii), the male fish liver maintains the functional ER-driven VTG response despite the fact it is not normally used; (iii), one of the main advantages of this system is that liver cells in culture maintain the endogenous receptor, activation factors and complete gene response systems so that any receptor cross-talk affecting the function of the ER can be detected; (iv), liver cells maintain complete xenobiotic metabolizing capability enabling detection of effects of estrogen metabolites; and (v), liver cells are able to detect both estrogen and anti-estrogen effects.

73. *In vitro* assays applied to screening of chemical including primary hepatocyte cultures and liver slice assays have been developed (*e.g.*, Shilling and Williams, 2000; Schmieder *et al.*, 2000, 2004; Navas and Segner, 2006). However, the main hurdles for regulatory acceptance of these assay systems seem to be the lack of standardised protocols for testing, minimizing the natural variability due to *e.g.* age, species, previous exposure, etc., when using primary cells. The test systems allow for the detection of chemically-induced ER agonism or antagonism by measuring VTG mRNA or protein by a variety of techniques (Navas and Segner, 2006, 2008).

> ***Recommendation 1:*** *There seem to be certain potential advantages by using sliced fish livers and primary hepatocyte cultures, however, since there are no available standardised test systems that have been evaluated or validated for regulatory purposes, it is recommended that existing assays and available data be further disseminated for possible additional recommendations towards validation studies, retrospective performance assessments or whether a draft Test Guideline can be developed. It should be noted as a point of concern that these assays consume fish and further precautions would have to be taken to minimize animal usage before a Test Guideline can be further developed, however, these assays have the potential of addressing two of the 3Rs, namely reduction and refinement.*

Yeast-Based Assays with Fish Steroid Hormone Receptors

74. The yeast-based YES and YAS (for AR) -assays have been recommended for use in monitoring

estrogenic and androgenic activity in contaminated environmental samples such as influent sewage water containing toxic compounds (Kinnberg, 2003) (see paragraph 67). However, it has been reported that the YES assay is not sensitive for anti-estrogenic chemicals (Fang *et al.,* 2000).

> ***Recommendation 2:*** *The YES/YAS-assays are recommended for further detailed evaluations primarily focusing on assays with fish steroid hormone receptors. It should be further evaluated whether such tests can provide meaningful information with special emphasis towards fish. Detailed comparisons on advantages/disadvantages to other in vitro assays, such as regarding yeast cell membrane permeability to certain compounds or chemical classes or other potential limitations, need to be clearly demonstrated before any further test method development or validation is performed. The sensitivity and specificity of any proposed YES/YAS assay needs to be demonstrated prior any further developments towards a Test Guideline.*

Assay- and Species Comparisons

75. Rider *et al.* (2009) developed a system using full-length recombinant baculovirus-expressed ERs which allows for direct comparison of ER-binding across different species. ERs representing five vertebrate classes were compared: hERα, quail ERα (qERα), alligator ERα (aERα), salamander ERα (sERα), and fathead minnow (fhmERα). Saturation binding analyses indicated E2 dissociation constants (Kd) as: 0.22 nM for hERα, 0.28 nM for sERα, 0.44 nM for aERα, 0.58 nM for qERα, and 0.58 nM for fhmERα. Binding specificity for each of the specified ERs was evaluated using E2, DHT, corticosterone (C), and EE2. E2 and EE2, and they were strong binders in all species with IC50s ranging from 0.65 nM with hERα to 1.01 nM with sERα for E2 and from 0.68 nM with sERα to 1.20 nM with qERα for EE2. DHT was a weak binder with IC50's ranging from 3.3 μM with hERα to 39 μM with fhERα, and C did not bind to any of the receptors at concentrations up to 100 μM. For receptor binding assays, recombinant proteins or extracted proteins from tissues can be used. TA assays are derived using fish tissues (*i.e.*, primary hepatocytes, tissue slices), transfected fish cell lines, or a variety of mammalian cell lines stably or transiently transfected with hormone receptor from fish and reporter genes that may or may not be derived from fish genes. Several studies presented in this DRP suggest evidence of species differences in affinity and sensitivity of hormone receptors (for a review see FIFRA-SAP 2009).

> ***Recommendation 3:*** *Receptor binding and TA assays using ER and AR from various fish species as well as those from other taxa should be directly compared in terms of their sensitivity, specificity and practicality considering different approaches. In addition, it should be evaluated whether there would be value added for assessment in multiple taxa. These assays should be applicable for the screening of a wide variety of chemicals of varying chemical classes and properties. As these in vitro assays are used more widely more information will become available for analysing the relationships between the in vitro and the in vivo assays for intra-, and inter-species comparisons and their potential regulatory utility. A majority of these assays are likely to be assays that parallel mammalian-based in vitro assays and the need for specific fish-receptor assays should be demonstrated prior to any development of Test Guidelines.*

Assay Performance Criteria

76. As with all assays, the *performance criteria* and the *testing conditions* under which the assays are conducted should be specified. For fish receptors and TA assays, it is particularly important to specify parameters that can vary significantly across assay systems such as *e.g.*, temperature. Hormone receptor binding and TA assays compare the response of the test chemical to that of a positive control, *e.g*. E2 for ER binding and ER-mediated TA assays. Thus the performance of the positive controls especially compared to historical values (where available) is useful in determining the reproducibility and reliability of the assay.

> ***Recommendation 4:***
>
> *To ensure applicability to a wide variety of chemicals with varying physical-chemical properties, it is recommended that chemicals should be tested up to solubility in the assay medium, or in whole cell or tissue-based assays up to cytotoxicity to determine an effect. In this case, the sensitivity of the specific assay is of less importance than the stability and reliability in relation to responses to specific endocrine mechanisms. If another criteria for determining maximum concentration in the assay system is used it should be specified to allow maximum comparability between systems. Any information available on measured chemical concentration, free fraction, or chemical metabolism in test systems is especially valuable when attempting to make comparisons across multiple systems and taxa. Future validation studies of fish receptor assays should ideally use chemicals based on results from existing validated OECD Test Guidelines to facilitate these comparisons.*

VII. REFERENCES

Ackermann, G.E., Brombacher, E. and Fent, K. (2002). Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents. *Environ. Toxicol. Chem.*, 21: 1864-1875.

Ankley, G.T., Defoe, D.L., Kahl, M.D., Jensen, K.M., Makynen, E.A., Miracle, A., Hartig, P., Gray, L.E., Cardon, M. and Wilson, V. (2004). Evaluation of the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol.*, 38: 6322-6327.

Ankley, G.T. and Villeneuve, D.L. (2006). The fathead minnow in aquatic toxicology: Past, present and future. *Aquat. Toxicol*., 78: 91–102.

Arukwe, A., Grotmol, T., Haugen, T.B., Knudsen, F.R. and Goksoyr, A. (1999). Fish model for assessing the *in vivo* estrogenic potency of the mycotoxin zearalenone and its metabolites. *Sci. Total Environ.*, 236: 153-161.

Barnes, K.K., Kolpin, D.W., Furlong, E.T., Zaugg, S.D., Meyer, M.T. and Barber, L.B. (2008). A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States--I) groundwater. *Sci. Total Environ.*, 402: 192-200.

Beck, I.C., Bruhn, R. and Gandrass, J. (2006). Analysis of estrogenic activity in coastal surface waters of the Baltic Sea using the yeast estrogen screen. *Chemosphere*, 63: 1870-1878.

Blumberg, B. and Evans, R.M., (1998). Orphan nuclear receptors—new ligands and new possibilities, *Genes Devlop.*, 12: 3149-3155.

Borg, B. (1994). Androgens in teleost fishes. *Comp. Biochem. Physiol.*, 109C: 219–245.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A. and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, 389: 753-758.

Dang, C.V., Barrett, J., Villa-Garcia, M., Resar, L.M., Kato, G.L. and Fearon, E.R. (1991) Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Mol. Cell Biol*., 11: 954-962.

Dang, Z. (2010). Comparison of relative binding affinities to fish and mammalian estrogen receptors: The regulatory implications. *Toxicol. Lett.*, 192: 298–315.

Denny, J.S., Tapper, M.A., Schmieder, P.K., Hornung, M.W., Jensen, K.M., Ankley, G.T. and Henry, T.R. (2005). Comparison of relative binding affinities of endocrine active compounds to fathead minnow and rainbow trout estrogen receptors. *Environ. Toxicol. Chem*., 24: 2948-2953.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P. and Waldock, M. (1999). Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and *In Vitro* Biological Screening. *Environ. Sci. Technol.*, 32:1549-1558.

Dietrich, D.R. and Krieger H. eds. (2009): Histological Analysis of Endocrine Disruptive Effects in Small

Laboratory Fish. ISBN: 978-0471763581, Wiley & Sons; Edition: 1., Edition (15. May 2009)

Donohoe, R.M. and Curtis, L.R. (1996). Estrogenic activity of chlordecone, *o,p*'-DDT and *o,p*'-DDE in juvenile rainbow trout: induction of vitellogenesis and interaction with hepatic estrogen binding sites. *Aquat. Toxicol*., 36: 31-52.

Dorizzi, M., Richard-Mercier, N., Desvages, G., Girondot, M. and Pieau, C. (1994). Masculization of gonads by aromatase inhibitors in a turtle with temperature-dependent sex determination, *Differentiation*, 58: 1-8.

Eidem, L.K., Kleivdal, H., Kroll, K., Denslow, N., van Aerle, R., Tyler, C., Panter, G., Hutchinson, T. and Grksoyr, A. (2006). Development and validation of a direct homologous quantitative sandwich ELISA for fathead minnow (*Pimephales promelas*) vitellogenin. *Aquat. Toxicol.*, 78: 202-206.

Elbrecht, A. and Smith, R.G. (1992). Aromatase enzyme activity and sex determination in chickens, *Science,* 255: 469–470.

Fang, H., Tong, W., Perkins, R., Soto, A.M., Prechtl, N.V. and Sheehan, D.M. (2000). Quantitative comparisons of in vitro assays for estrogenic activities. *Environ. Health Perspect.*, 108: 723–729

Fearon, E.R., Finkel, T., Gillison, M.L., Kennedy, S.P., Casella, J.F., Tomaselli, G.F., Morrow, J.S. and Van Dang, C. (1992). Karyoplasmic interaction selection strategy: a general strategy to detect protein-protein interactions in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.*, 89: 7958-7962.

Fent, K. (2001). Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicol. In Vitro*, 15: 477-488.

FIFRA-SAP (2009). US Federal Insecticide, Fungicide, and Rodenticide (FIFRA) Scientific Advisory Panel (SAP) Meeting on August 25-26, 2009. The use of structure activity relationships of estrogen affinity to support prioritization of pesticide inert ingredients and antimicrobial pesticides for screening and testing. Meeting minutes available: [http://www.epa.gov/scipoly/sap/meetings/2009/august/082509minutes.pdf]

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B. and Thurman, M.E. (2008). A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States--II) untreated drinking water sources. *Sci. Total. Environ.*, 402: 201-16.

Folmar, L.C., Hemmer, M.J., Denslow, N.D., Kroll, K., Chen, J., Cheek, A., Richman, H., Meredith, H. and Grau, E.G. (2002). A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor *in vivo* and *in vitro*. *Aquat. Toxicol.,* 60: 101-110.

Gagné, F. and Blaise, C. (1998). Estrogenic properties of municipal and industrial wastewaters evaluated with a rapid and sensitive chemoluminescent *in situ* hybridization assay (CISH) in rainbow trout hepatocytes. *Aquat. Toxicol*., 44: 83–91.

Gale, W.L. Patino, R. and Maule, A.G. (2004). Interaction of xenobiotics with estrogen receptors α and β and a putative plasma sex hormone binding globulin from channel catfish (Ictalurus punctatus). *Gen. Comp. Endocrinol.,* 136: 338-345.

Gray, L.E.Jr., Ostby, J., Wilson, V., Lambright, C., Bobseine, K., Hartig, P., Hotchkiss, A., Wolf, C., Furr, J., Price, M., Parks, L., Cooper, R.L., Stoker, T.E., Laws, S.C., Degitz, S.J., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., Tietge, J.E. and Ankley, G.T. (2002). Xenoendocrine disrupters-tiered screening and testing: filling key data gaps. *Toxicology,* 181-182: 371-382.

Green, S., Walter, P., Kumer, V., Krust, A., Bornet, J.M., Argos, P. and Chambon, P. (1986). Human oestrogen receptor cDNA: sequence expression and homology to v-*erb*-A, *Nature,* 320: 134–139.

Guevel, R.L., Petit, F.G., Goff, P.L., Metivier, R., Valotaire, Y. and, Pakdel, F. (2000). Inhibition of rainbow trout (*Oncorhynchus mykiss*) estrogen receptor activity by cadmium. *Biol. Reprod.,* 63: 259-266.

Guillette, L.J.Jr., Arnold, S.F. and McLachlan, J.A. (1996). Ecoestrogens and embryos—is there a scientific basis for concern?, *Anim. Reprod. Sci.,* 42: 13–24.

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A. and Thomas, P. (2000). Identification of a third estrogen receptor and reclassification of estrogen receptors in teleosts, *Proc. Natl. Acad. Sci. U.S.A.,* 97: 10751–10756.

Heringa, M.B., Schreurs, R.H., Busser, F., van der Saag, P.T., van der Burg, B. and Hermens, J.L. (2004). Toward more useful *in vitro* toxicity data with measured free concentrations.*Environ. Sci. Technol.,* 38: 6263-6270.

Hornung, M.W., Ankley, G.T. and Schmieder, P.K. (2003). Induction of an estrogen-responsive reporter gene in rainbow trout hepatoma cells (RTH 149) at 11 or 18 degrees C. *Environ. Toxicol. Chem.*, 22: 866-871.

Hornung, M.W., Jensen, K.M., Korte, J.J., Kahl, M.D., Durhan, E.J., Denny, J.S., Henry, T.R. and Ankley, G.T. (2004). Mechanistic basis for estrogenic effects in fathead minnow (*Pimephales promelas*) following exposure to the androgen 17α-methyltestosterone: conversion of 17α-methyltestosterone to 17α-methylestradiol. *Aquat. Toxicol*., 66: 15-23.

Huggett, D.B., Foran, C.M., Brooks, B.W., Weston, J., Peterson, B., Marsh, K.E., La Point, T.W. and Schlenk, D. (2003). Comparison of *in vitro* and *in vivo* bioassays for estrogenicity in effluent from North American municipal wastewater facilities. *Toxicol. Sci.,* 72: 77-83.

Hutchinson, T.H. (2002). Reproductive and developmental effects of endocrine disrupters in invertebrates: *in vitro* and *in vivo* approaches. *Toxicol. Lett.,* 131: 75-81.

Hutchinson, T.H. (2007). Small is useful in endocrine disrupter assessment four key recommendations for aquatic invertebrate research. *Ecotoxicology,* 16: 231-238.

Iguchi, T., Ohta, Y., Fukazawa, Y. and Takasugi, N. (1987). Strain differences in the induction of polyovular follicles by neonatal treatment with diethylstilbestrol in mice. *Med. Sci. Res.,* 15: 1407-1408.

Iguchi, T., Irie, F., Urushitani, H., Tooi, O., Kawashima, Y., Roberts, M., Norrgren, L. and Hutchinson, T.H. (2006a). Availability of *in vitro* vitellogenin assay for screening of estrogenic and anti-estrogenic activities of environmental chemicals. *Environ. Sci*., 13: 161-183.

Iguchi, T., Watanabe, H. and Katsu, Y. (2001). Developmental effects of estrogenic agents on mice, fish and frogs: a mini-review, *Horm. Behav.,* 40: 248–251.

Iguchi, T., Watanabe, H. and Katsu, Y. (2006b). Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. *Environ. Health Perspect*., 114 Suppl.1: 101-105..

Iguchi, T., Watanabe, H. and Katsu, Y. (2007). Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology in vertebrates and invertebrates. *Gen. Comp. Endocrinol*., 153: 25-29.

Ikeuchi, T., Todo, T., Kobayashi, T. and Nagahama,Y. (1999). cDNA cloning of a novel androgen receptor subtype. *J. Biol. Chem.,* 274: 25205–25209.

Islinger, M., Pawlowski, S., Hollert, H., Volkl, A. and Braunbeck, T. (1999). Measurement of vitellogenin mRNA expression in primary cultures of rainbow trout hepatocytes in a non-radioactive dot blot/RNAse protection assay. *Sci. Total Environ.,* 233: 109–122.

Jena, N.R., Sarkar, S., Yonemoto, J., Tohyama, C. and Sone, H. (1998). Strain differences in cytochrome P4501A1 gene expression caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat liver: role of the aryl hydrocarbon receptor and its nuclear translocator. *Biochem. Biophys. Res. Commun*., 248: 554-558.

Jobling, S. and Sumpter, J.P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat. Toxicol.*, 27: 361–372.

Jobling, S., Reynolds, T., White, R., Parker, M.G. and Sumpter, J.P. (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ. Health Perspect*., 103: 582-587.

Karchner, S.I., Franks, D.G., Kennedy, S.W. and Hahn, M.E. (2006). The molecular basis for differential dioxin sensitivity in birds: role of the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci. U.S.A.*, 103: 6252-6257.

Kato, Y., Kobayashi, K., Oda, S., Tatarazako, N., Watanabe, H. and Iguchi, T. (2007). Cloning and characterization of the ecdysone receptor and ultraspiracle protein from the water flea *Daphnia magna*. *J. Endocrinol*., 193: 183-194.

Katsu, Y., Bermudez, D.S., Braun, E., Helbing, C., Miyagawa, S., Gunderson, M.P., Kohno, S., Bryan, T.A., Guillette, L.J. Jr. and Iguchi, T. (2004). Molecular cloning of the estrogen and progesterone receptors of the American alligator, *Gen. Comp. Endocrinol.*, , 136: 122–133.

Katsu, Y., Myburgh, J., Kohno, S., Swan, G.E., Guillette, L.J.Jr. and Iguchi, T. (2006a). Molecular cloning of estrogen receptor α of the Nile crocodile. *Comp. Biochem. Physiol., Part A*, 143: 340-346.

Katsu, Y., Kohno, S., Oka, T., Mitsui, N., Tooi, O., Santo, N., Urushitani, H., Fukumoto, Y., Kuwabara, K., Ashikaga, K., Minami, S., Kato, S., Ohta, Y., Guillette, L.J.Jr. and Iguchi, T. (2006b). Molecular cloning of estrogen receptor alpha (ERα; ESR1) of the Japanese giant salamander, *Andrias japonicus. Mol. Cell. Endocr.*, 257-258: 84-94.

Katsu, Y., Lange, A., Ichikawa, R., Urushitani, H., Paull, G.C., Cahill, L.L., Jobling, S., Tyler, C.R. and Iguchi, T. (2007a). Functional associations between two estrogen receptors, environmental estrogen and sexual disruption in the roach (*Rutilus rutilus*). *Environ. Sci. Technol*., 41: 3360-3374.

Katsu, Y., Hinago, M., Sone, K., Guillette, L.J.Jr. and Iguchi, T. (2007b). Analysis the ligand-specificity of the estrogen and androgen receptors of mosquitofish, *Gambusia affinis affinis. Mol. Cell. Endocr.*, 276: 10-

17.

Katsu, Y., Ichikawa, R., Ikeuchi, T., Kohno, S., Guillette, L.J.Jr. and Iguchi, T. (2008). Molecular cloning and characterization of estrogen, androgen and progesterone nuclear receptors from a freshwater turtule (*Pseudemys nelsoni*). *Endocrinology*, 149: 161-173.

Kavlock, R.J. and Ankley, G.T. (1996). A perspective on the risk-assessment process for endocrine-disruptive effects on wildlife and human health. *Risk Analysis*, 16: 731-739.

Kawakami, T., Ishimura, R., Nohara, K., Takeda, K., Tohyama, C. and Ohsako, S. (2006). Differential susceptibilities of Holtzman and Sprague-Dawley rats to fetal death and placental dysfunction induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) despite the identical primary structure of the aryl hydrocarbon receptor. *Toxicol. Appl. Pharmacol.*, 212: 224-236.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M. and Flick, R.W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. U.S.A.*, 104: 8897-8901.

Kinnberg, K. (2003). Evaluation of *in vitro* assays for determination of estrogenic activity in the environment. *Working Report No. 43, Danish Environmental Protection Agency*.

Kloas, W., Schrag, B., Ehnes, C. and Segner, H. (2000). Binding of xenobiotics to hepatic estrogen receptor and plasma sex steroid binding protein in the teleost fish, the common carp (*Cyprinus carpio*). *Gen. Comp. Endocrinol*., 119: 287-299.

Knudsen, F.R. and Pottinger, T.G. (1999). Interaction of endocrine disrupting chemicals, singly and in combination, with estrogen-, androgen-, and corticosteroid-binding sites in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.*, 44: 159-170.

Koike, S., Sakai, M. and Muramatsu, M. (1987). Molecular cloning and characterization of rat estrogen receptor cDNA, *Nucl. Acids Res.*, 15: 2499–2513.

Kolpin, D.W., Furlong, E.T., Meyer, M T., Thurman, E.M., Zaugg, S.D., Barber, L.B., and Buxton, H.T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: A national reconnaissance. *Environ. Sci. Tech.*, 36: 1202–1211.

Körner, W., Vinggaard, A.M., Terouanne, B., Ma, R., Wieloch, C., Schlumpf, M., Sultan, C. and Soto, A.M. (2004). Interlaboratory comparison of four *in vitro* assays for assessing androgenic and antiandrogenic activity of environmental chemicals. *Environ. Health Perspect.*, 112: 695-702.

Krust, A., Green, S., Argos, P., Bumar, V., Walter, J.M.B.andChambon, P. (1986). The chicken oestrogen receptor sequence: homology with v-*erb*-A and the human oestrogen and glucocorticoid receptor, *EMBO J.*, 5**:** 891–897.

Kurauchi, K., Nakaguchi, Y., Tsutsumi, M., Hori, H., Kurihara, R., Hashimoto, S., Ohnuma, R., Yamamoto, Y., Matsuoka, S., Kawai, S., Hirata, T. and Kinoshita, M. (2005). *In vivo* visual reporter system for detection of estrogen-like substances by transgenic medaka. *Environ. Sci. Technol*., 39: 2762-2768.

Kwon, H.C., Hayashi, S. and Mugiya, Y. (1993). Vitellogenin induction by estradiol-17β in primary hepatocyte culture in the rainbowtrout *Oncorhynchus mykiss*. *Comp. Biochem. Physiol.*, 104B: 381–386.

Legler, J., Zeinstra, L.M., Schuitemaker, F., Lanser, P.H., Bogerd, J., Brouwer, A., Vethaak, A.D., De Voogt, P., Murk, A.J. and Van der Burg, B. (2002). Comparison of *in vivo* and *in vitro* reporter gene assays for short-term screening of estrogenic activity. *Environ. Sci. Technol*., 36: 4410-4415.

Leihy, M.W., Shaw, G., Wilson, J.D. and Renfree, M.B. (2004). Penile development is initiated in the tammar wallaby pouch young during the period when 5α-androstane-3 α,17β-diol is secreted by the testes. *Endocrinology,* 145: 3346–3352.

Link, V., Shevchenko, A. and Heisenberg, C.P. (2006). Proteomics of early zebrafish embryos. *BMC Dev. Biol.*, 6:1.

Long, X., Steinmetz, R., Ben-Jonathan, N., Caperell-Grant, A., Young, P.C., Nephew, K.P. and Bigsby, R.M. (2000). Strain differences in vaginal responses to the xenoestrogen bisphenol A. *Environ. Health Perspect*., 108: 243-247.

Loomis, A.K. and Thomas, P. (1999). Binding characteristics of estrogen receptor (ER) in Atlantic croaker (*Micropogonias undulatus*) testis: different affinity for estrogens and xenobiotics from that of hepatic ER. *Biol. Reprod*., 61: 51-60.

Matthews, J., Celius, T., Halgren, R. and Zacharewski, T. (2000). Differential estrogen receptor binding of estrogenic substances: a species comparison. *J. Steroid Biochem. Mol. Biol*., 74: 223-234.

Matthews, J., Fertuck, K.C., Celius, T., Huang, Y-W., Fong, C.J. and Zacharewski, T.R. (2002). Ability of structurally diverse natural products and synthetic chemicals to induce gene expression mediated by estrogen receptors from various species. *J. Steroid Biochem. Mol. Biol*., 82: 181-194.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O. and Pakdel, F. (2002). Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biol. Reprod.*, 66: 1881-1892.

Murk, A.J., Legler, J., Denison, M.S., Giesy, J.P., van de Guchte, C. and Brouwer, A. (1996). Chemical-activated luciferase gene expression (CALUX): a novel *in vitro* bioassay for Ah receptor active compounds in sediments and pore water. *Fund. Appl. Toxicol*., 33: 149-160.

Nakai, M. (2003). Receptor binding assay and reporter gene assay of medaka. In Medaka *Oryzias latipes*. *Ministry of the Environment, Japan,* pp. 21-26. Available at: http://www.env.go.jp/chemi/end/pdfs/e06_chapter2.pdf.

Nakai,, M., Yokota,, H., Urushitani,, H., Asai, D,, Katsu, Y., Eto, C., Yakabe, Y. Iguchi, T. and Shimohigashi, Y. (2004). Estrogen receptor binding assay for evaluation of endocrine disrupting chemicals on marine fish. *Mar. Biotechnol*., 6: S137-S141.

Navas, J.M. and Segner, H. (2006). Vitellogenin synthesis in primary cultures of fish liver cells as endpoint for *in vitro* screening of the (anti)estrogenic activity of chemical substances. *Aquat. Toxicol*., 80: 1-22.

Navas, J.M. and Segner, H. (2008). In vitro screening of the antiestrogenic activity of chemicals. *Exp. Opin. Drug Metab. Toxicol.*, 4: 605-617.

Nimrod, A.C. and Benson, W.H. (1997). Xenobiotic interaction with and alteration of channel catfish estrogen receptor. *Toxicol. Appl. Pharmacol*., 147: 381-90.

Nishizuka, M., Heitaku, S., Maekawa, S., Nishikawa, J. and Imagawa. M. (2004). Development of standardized *in vitro* assay system to estrogen receptors and species specificity of binding ability of 4-nonylphenol and *p*-octylphenol. *J. Health Sci*., 50: 511-517.

Norris, D.O. (1996). *Vertebrate Endocrinology*. Adademic Press, San Diego, CA, USA,. ISBN: 012521670X.

OECD (2007), Uterotrophic Bioassay in Rodents: A short-term screening test for oestrogenic properties No. 440. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

OECD (2008), Repeated Dose 28-Day Oral Toxicity Study in Rodents No. 407. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

OECD (2009a), Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals No. 455. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

OECD (2009b), Hershberger Bioassay in Rats: A Short-term Screening Assay for (Anti)Androgenic Properties No. 441. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

OECD (2009c), 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition No. 230. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines].

Ogino, Y., Katoh, H. and Yamada, G. (2004) Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. *FEBS Lett.*, 575: 119–126.

Ogino, Y., Katoh, H., Kuraku, S. and Yamada, G. (2009). Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. *Endocrinology,* 150: 5415–5427.

Okoumassoun, L-E., Averill-Bates, D., Gagné, F., Marion, M. and Denizeau, F. (2002). Assessing the estrogenic potential of organochlorine pesticides in primary cultures of male rainbow trout (*Oncorhynchus mykiss*) hepatocytes using vitellogenin as a biomarker. *Toxicology* 178: 193–207.

Olsen, C.M., Meussen-Elholm, E.T.M., Hongslo, J.K., Stenersen, J. and Tollefsen, K-E. (2005). Estrogenic effects of environmental chemicals: an interspecies comparison. *Comp. Biochem. Physiol.*, 141C: 267–274.

Örn, S., Yamani, S. and Norrgren, L. (2006). Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17α-ethinylestradiol and 17β-trenbolone. *Arch. Environ. Contam. Toxicol*., 51: 237-243.

Pakdel, F., Le Gac, F., Le Goff, P. and Valotaire, Y. (1990). Full-length sequence and *in vitro* expression of rainbow trout estrogen receptor cDNA, *Mol. Cell. Endocrinol.*, 71: 195–204.

Paris, F., Balaguer, P., Térouanne, B., Servant, N., Lacoste, C., Cravedi, J.P., Nicolas, J.C. and Sultan, C. (2002). Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines. *Mol. Cell. Endocrinol*., 193: 43-49.

Parks, L.G., Lambright, C.S., Orlando, E.F., Guillette, L.J. Jr., Ankley, G.T. and Gray, L.E. Jr. (2001). Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol. Sci.*, 62: 257-267.

Pelissero, C., Flouriot, G., Foucher, J.L., Bennetau, B., Dunogues, J., Le Gac, F. and Sumpter, J.P. (1993). Vitellogenin synthesis in cultured hepatocytes; an *in vitro* test for the estrogenic potency of chemicals. *J. Steroid Biochem. Mol. Biol.*, 44: 263–272.

Petit, F., Le Goff, P., Cravedi, J.P., Valotaire, Y. and Pakdel, F. (1997). Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. *J. Mol. Endocrinol.*, 19: 321-335.

Pillon, A., Boussioux, A.M., Escande, A., Aït-Aïssa, S., Gomez, E., Fenet, H., Ruff, M., Moras, D., Vignon, F., Duchesne, M.J., Casellas, C., Nicolas, J.C. and Balaguer, P. (2005). Binding of estrogenic compounds to recombinant estrogen receptor-alpha: application to environmental analysis. *Environ. Health Perspect.*, 113: 278-284.

Quigley, C.A., De Bellis, A., Marschke, K.B., el-Awady, M.K., Wilson, E.M. and French, F.S. (1995). Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr. Rev.*, 16: 271–321.

Rankouhi, T.R., Sanderson, J.T., van Holsteijn, I., van Leeuwen, C., Vethaak, A.D. and van den Berg, M. (2004). Effects of natural and synthetic estrogens and various environmental contaminants on vitellogenesis in fish primary hepatocytes: comparison of bream (*Abramis brama*) and carp (*Cyprinus carpio*). *Toxicol. Sci*., 81: 90–102.

Rider CV, Hartig PC, Cardon MC, Wilson VS.: Development of a competitive binding assay system with recombinant estrogen receptors from multiple species. *Toxicol. Lett.*, 184: 85-89, 2009a.

Rider, C.V., Hartig, P.C., Cardon, M.C. andd Wilson, V.S. (2009a). Comparison of chemical binding to recombinant fathead minnow and human estrogen receptors alpha in whole cell and cell-free binding assays. *Environ. Toxicol. Chem.,* 28: 2175-2181.

Rider, C.V., Hartig, P.C., Cardon, M.C. and Wilson, V.S. (2009b). Development of a competitive binding assay system with recombinant estrogen receptors from multiple species. *Toxicol. Lett.*, 184: 85-89.
Rotchell, J.M. and Ostrander, G.K. (2003). Molecular markers of endocrine disruption in aquatic organism. *J. Toxicol. Environ. Health B Crit. Rev*., 6: 453-496.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M., and Sumpter, J.P. (1998). Identification of estrogenic chemicals in STW effluent: 2. *In vivo* responses in trout and roach. *Environ. Sci. Tech.,* 32: 1559–1565.

Routledge, E.J. and Sumpter, J.P. (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem*., 15: 241-248.

Schmieder, P., Tapper, M., Linnum, A., Denny, J., Kolanczyk, R. and Johnson, R. (2000). Optimization of a precision-cut trout liver tissue slice assay as a screen for vitellogenin induction: comparison of slice incubation techniques. *Aquat. Toxicol*., 49: 251-268.

Schmieder, P.K., Tapper, M.A., Denny, J.S., Kolanczyk, R.C., Sheedy, B.R., Henry, T.R. and Veith, G.D. (2004). Use of trout liver slices to enhance mechanistic interpretation of estrogen receptor binding for cost-effective prioritization of chemicals within large inventories. *Environ. Sci. Technol.*, 38: 6333-6342.

Scholz, S., Kordes, C., Hamann, J. and Gutzeit, H.O. (2004). Induction of vitellogenin *in vivo* and *in vitro* in the model teleost medaka (*Oryzias latipes*): comparison of gene expression and protein levels. *Marine*

Environ. Res., 57: 235-244.

Scholz, S., Kurauchi, K., Kinoshita, M., Oshima, Y., Ozato, K., Schirmer, K. and Wakamatsu, Y. (2005). Analysis of estrogenic effects by quantification of green fluorescent protein in juvenile fish of a transgenic medaka. *Environ. Toxicol. Chem*., 24: 2553-2561.

Schreurs, R.H., Quaedackers, M.E., Seinen, W. and van der Burg, B. (2002). Transcriptional activation of estrogen receptor ERalpha and ERbeta by polycyclic musks is cell type dependent. *Toxicol. Appl. Pharmacol.,* 183: 1-9.

Schreurs, R.H., Legler, J., Artola-Garicano, E., Sinnige, T.L., Lanser, P.H., Seinen, W. and Van der Burg, B. (2004). *In vitro* and *in vivo* antiestrogenic effects of polycyclic musks in zebrafish. *Environ. Sci. Technol.,* 38: 997-1002.

Seki, M., Fujishima, S., Nozaka, T., Maeda, M. and Kobayashi, K. (2006). Comparison of response to 17β-estradiol and 17β-trenbolone among three small fish species. *Environ. Toxicol. Chem*., 25: 2742-2752.

Shilling, A. and Williams, D. (2000). Determining relative estrogenicity by quantifying vitellogenin induction in rainbow trout liver slices. *Toxicol. Appl. Pharmacol*., 164: 330-335.

Smith, J.S. and Thomas, P. (1990). Binding characteristics of the hepatic estrogen receptor of the spotted sea trout, *Cynoscion nebulosus*. *Gen. Comp. Endocrinol*., 77: 29-42.

Sone, K., Hinago, M., Itamoto, M., Katsu, Y., Watanabe, H., Urushitani, H., Tooi, O., Guillette, L.J. Jr. and Iguchi, T. (2005). Effects of an androgenic growth promoter 17β-trenbolone on masculinization of mosquitofish (*Gambusia affinis affinis*). *Gen. Comp. Endocrinol*., 143: 151-60.

Soto, A.M., Maffini, M.V., Schaeberle, C.M. and Sonnenschein, C. (2006). Strengths and weaknesses of *in vitro* assays for estrogenic and androgenic activity. *Best Pract. Res. Clin. Endocrinol. Metab.*, 20: 15-33.

Spearow, J.L., Doemeny, P., Sera, R., Leffler, R. and Barkley, M. (1999). Genetic variation in susceptibility to endocrine disruption by estrogen in mice. *Science*, 285: 1259-1261.

Sperry, T.S. and Thomas, P. (1999). Characterization of two nuclear androgen receptors in *Atlantic croaker*: comparison of their biochemical properties and binding specificities. *Endocrinology,* 140: 1602–1611.

Sumida, K., Ooe, N., Saito, K. and Kaneko, H. (2001). Molecular cloning and characterization of reptilian estrogen receptor cDNAs, *Mol. Cell. Endocrinol.,* 183: 33–39.

Sumida, K., Ooe, N., Saito, K. and Kaneko, H. (2003). Limited species differences in estrogen receptor alpha-mediated reporter gene transactivation by xenoestrogens. *J. Steroid Biochem. Mol. Biol*., 84: 33-40.

Sumpter, J.P. and Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.,* 103 (Suppl. 7): 173-178.

Takeo, J. and Yamashita, S. (1999). Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. *J. Biol. Chem*., 274: 5674-5680.

Takeo, J. and Yamashita, S. (2000). Rainbow trout androgen receptor-α fails to distinguish between any of the natural androgens tested in transactivation assay, not just 11-ketotestosterone and testosterone. *Gen. Comp. Endocrinol*., 117: 200-206.

Thomas, P. and Smith, J. (1993). Binding of xenobiotics to the estrogen receptor of spotted sea trout: A screening assay for potential estrogenic effects. *Mar. Environ. Res*., 35: 147-151.

Thornton, J.W. (2001). Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions, *Proc. Natl. Acad. Sci. U.S.A.*, 98: 5671–5676.

Todo, T., Ikeuchi, T., Kobayashi, T. and Nagahama, Y. (1999). Fish androgen receptor: cDNA cloning, steroid activation of transcription in transfected mammalian cells, and tissue mRNA levels. *Biochem. Biophys. Res. Commun.*, 254: 378–383.

Tollefsen, K.E., Mathisen, R. and Stenersen, J. (2002). Estrogen mimics bind with similar affinity and specificity to the hepatic estrogen receptor in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol*., 126: 14-22.

Tyler, C.R., Jobling, S. and Sumpter, J.P. (1998). Endocrine disruption in wildlife: a critical review of the evidence. *Crit. Rev. Toxicol*., 28: 319-361.

Urushitani, H., Nakai, M., Inanaga, H., Shimohigashi, Y., Shimizu, A., Katsu, Y. and Iguchi, T. (2003). Cloning and characterization of estrogen receptor α in mummichog, *Fundulus heteroclitus*. *Mol. Cell. Endocrinol*., 203: 41-50.

Vaillant, C., Le Guellec, C., Pakdel, F. and Valotaire, Y. (1988). Vitellogenin gene expression in primary culture of male rainbowtrout hepatocytes. *Gen. Comp.Endocrinol*., 70, 284–290.

van Der Kraak, G.J., Zacharewski, T., Janz, D., Sanders, B. and Gooch, J. (1998). Comparative endocrinology and mechanisms of endocrine modulation in fish and wildlife. In Kendall R, Dickson R, Suk W, Giesy J, eds. *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*. SETAC Press, Pensacola, FL, USA, pp. 97-120.

Wallace, R.A. (1985). Vitellogenesis and oocyte growth in non-mammalian vertebrates. In: L.W. Browder, Editor, *Development Biology*, Plenum Press, New York, pp. 127–177.

Watanabe, H., Takahashi, E., Nakamura, Y., Oda, S., Tatarazako, N. and Iguchi, T. (2007). Development of *Daphnia magna* DNA microarray for the evaluation of toxicity of environmental chemicals. *Environ. Toxicol. Chem*., 26: 669-676.

Watanabe, H., Tatarazako, N., Oda, S., Nishide, H., Uchiyama, I., Morita, M. and Iguchi, T. (2005). Analysis of expressed sequence tags of the water flea *Daphnia magna*. *Genome*, 48: 606-609.

Weiler, I.J., Lew, D. and Shapiro, D.J. (1987). The *Xenopus laevis* estrogen receptor: sequence homology with human and avian receptors and identification of multiple estrogen receptor messenger ribonucleic acids, *Mol. Endocrinol.*, 1: 355–362.

White, R., Lees, J.A., Needham, M., Ham, J. and Parker, M. (1987). Structural organization and expression of the mouse estrogen receptor, *Mol. Endocrinol.*, 1: 735–744.

Wilson, V.S., Bobseine, K. and Gray, L.E. Jr. (2004a). Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol. Sci*., 81: 69-77.

Wilson, V.S., Cardon, M.C., Gray, L.E.Jr. and Hartig, P.C. (2007). Comparative binding comparison of endocrine-disrupting compounds to recombinant androgen receptor from fathead minnow, raibow trout, and human. *Environ. Toxicol. Chem.*, 26: 1793–1802.

Wilson, V.S., Cardon, M.C., Thornton, J., Korte, J.J., Ankley, G.T., Welch, J., Gray, L.E. Jr. and Hartig, P.C. (2004b). Cloning and *in vitro* expression and characterization of the androgen receptor and isolation of estrogen receptor from the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol*., 38: 6314-6321.

Table 1. Receptor Binding Assays

Type/isoform	Source	Tissue		Species	Chemicals: classes tested	Chemicals: No. of chem tested	REFS	paragraph
ER	1) Endogenous							
mixed		liver	a) cytosol	rainbow trout	phthalates, benzophenones, subst benzenes BHA, subst.phenols benzoic acids	20	Jobling *et al.*, 1995	22
mixed		liver	a) cytosol	rainbow trout	steroids alkylphenols phytoestrogen	11	Denny *et al.*, 2005	23
mixed		liver	a) cytosol	rainbow trout	steroids pharmaceuticals pesticides alkylphenols	16	Schmieder *et al.*, 2004	24
mixed		liver	a) cytosol	rainbow trout	alkylphenols phthalates pesticides phytoestrogen	18	Knudsen and Pottinger, 1999	25
mixed		liver	a) cytosol	rainbow trout	phytoestrogen	3	Arukwe *et al.*, 1999	26
mixed		liver	a) cytosol	rainbow trout	steroids pharmaceuticals phytotoxins/mycotoxins pesticides alkylphenols	15	Tollefsen *et al.*, 2002	27
mixed			a) cytosol	rainbow trout	Chlordecone, *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE	4	Donohoe and Curtis, 1996	29
mixed		liver, ovary, heart, spleen, muscle, brain	a) cytosol b) nucleus	spotted sea trout	potent estrogens	14	Smith and Thomas, 1990	34
mixed		liver	a) cytosol	channel catfish	steroids pharmaceuticals pesticides	13	Nimrod and Benson, 1997	46

					alkylphenol DDT, DDEs			
mixed		liver, testicular tissue	a) cytosol b) nucleus	atlantic croaker	steroids pharmaceuticals pesticides alkylphenol DDT, DDEs PCBs	29	Loomis and Thomas, 1999	48
mixed		liver	a) cytosol	atlantic salmon	steroids pharmaceuticals phytotoxins/mycotoxins pesticides alkylphenols	15	Tollefsen *et al.*, 2002	49
mixed		liver	a) cytosol	carp	steroids pharmaceuticals Industrial chemicals pesticides	22	Kloas *et al.*, 2000	51
	2)Recombinant Expression cells	receptor details		ER species origin				
ER alpha	E. coli	LBD		rainbow trout	steroids alkyphenols mycotoxins pesticides phytoestrogens	34	Matthews *et al.*, 2000	28
ER alpha	E. coli	full length		rainbow trout	heavy metals	6	Guevel *et al.*, 2000	33
ER alpha	yeast cells	LBD		mummichog	alkylphenols phthalates styrene TBT	8	Urushitani *et al.*, 2003	36
ER alpha beta	E. coli	LBD		medaka	alkylphenols phthalates styrene TBT	13	Nakai *et al.*, 2003	37

ER alpha beta	E. coli	LBD		medaka	pharmaceuticals alkylphenols	3	Nishizuka *et al.*, 2004	38
ER alpha beta	COS-7 cell	full		channel catfish	steroids alkylphenols dieldrin endosulfan	9	Gale *et al.*, 2004	47
ER alpha	E. coli	LBD		red sea bream	alkyl phenols	3	Nakai *et al.*, 2003	52
AR	1) Endogenous							
	2) cytosol	brain	a)cytosol	rainbow trout	phthalates pesticides	6	Knudsen and Pottinger, 1999	25
	2)Recombinant Expression cells	receptor details		AR species origin				
AR alpha	COS-1	full		rainbow trout	steroids	5	Takeo and Yamashita, 2000	58
AR alpha	COS	full		fathead minnow	steroids pharmaceuticals anti-androgens	14	Wilson *et al.*, 2004b	59
AR alpha	COS-1	full		fathead minnow	flutamides	2	Ankley *et al.*, 2004	60

Table 2. Transactivation Assays

Type/isoform	Source	Tissue	reporter details	Species	Chemicals		References	Comments	paragraph
					classes tested	No. of chem tested			
ER	1) Endogenous								
mixed		Tissue Slices		rainbow trout	steroids pharmaceuticals pesticides alkylphenols	3	Schmieder et al., 2004		17
	2) recombinant CELL TYPE	receptor details							
ER alpha	RTG-2	a) full length	a) luciferase	rainbow trout	alkylphenols DDT-isoforms and metabolites	12	Fent, 2001		31
ER alpha	RTG-2	a) full length	a) luciferase	rainbow trout	alkyphenols DDTs	12	Ackermann *et al.*, 2002		31
ER alpha	RTH-149	a) full length	a) luciferase	rainbow trout	EE2 penthylphenol	2	Hornung *et al.*, 2003	temp sens of rbtER	32
ER alpha beta	HeLa	a) full length	a) luciferase	medaka	alkyphenols phthalates TBT OCS	13	Nakai *et al.*, 2003		37
ER alpha beta1 beta2	CHO	a) full length	a) luciferase	zebrafish	steroids pharmaceuticals	10	Menuet *et al.*, 2002		43
ER alpha beta	HEK293	a) full length	a) luciferase	zebrafish	steroids DDTs	6	Legler *et al.*, 2002		44

gamma					nonylphenol			
ER alpha beta	CHO-K1	a) full length	a) luciferase	roach	steroids pharmaceuticals alkylphenols	6	Katsu *et al.*, 2007a	53
ER alpha beta	HEK293	a) full length	a) luciferase	mosquitofish	steroids	4	Katsu *et al.*, 2007b	54
ER alpha	HeLa BF-2	a) full length	a) luciferase	rainbow trout	steroids pharmaceuticals alkylphenols phytoestrogens DDT	10	Sumida *et al.*, 2003	56
AR	recombinant CELL TYPE	receptor details						
	HeLa	a) full length	a) luciferase	medaka	alkylphenols phthalates TBT OCS	13	Nakai *et al.*, 2003	37
AR alpha beta	HEPG2	a) full length	a) luciferase	mosquitofish	steroids	6	Katsu *et al.*, 2007b	54
AR alpha	EPC	a) full length	b) CAT	rainbow trout	steroids	8	Takeo and Yamashita, 2000	58

Table 3. Results of medaka estrogen receptors (α and β) binding assay, medaka estrogen receptor transactivation assay, medaka androgen receptor transactivation assay, and medaka 21-day vitellogenin (VTG) assay.

Chemicals	Estrogen receptor α (%)		Estrogen receptor β (%)		Androgen receptor (%)	21-day VTG assay Lowest concentration induced VTG in male medaka
	Binding assay	Transactivation assay	Binding assay	Transactivation assay	Transactivation assay	
17β-Estradiol	100	100	100	100		21.9 ng/L
Dihydrotestosterone					100	
Ethynylestradiol	78	97	80	226	5.9	14.8 ng/L
4-*t*-Octylphenol	16	1.3	0.83	-	-	64.1 μg/L
4-Nonylphenol (branched)	8.1	0.35	0.83	-	-	22.5 μg/L
Di-(2-ethylhexyl) phthalate	0.79	-	0.37	-	-	Not induced
trans-Nanochlor	0.60	ND	0.022	-	-	Not induced
o,p'-DDT	0.54	ND	0.17	-	-	1.5 μg/L
Bisphenol A	0.48	0.076	0.31	-	-	334 μg/L
cis-Chlordane	0.31	ND	0.22	-	-	Not induced
Triphenyltin chloride	0.24	-	0.29	-	-	Not induced
Butyl benzyl phthalate	0.23	ND	0.095	ND	-	Not induced
Tributyltin chloride	0.14	-	0.19	-	-	Not induced
p,p'-DDT	0.12	ND	0.069	-	-	Not induced
Dicyclohexyl phthalate	0.045	-	0.016	-	-	Not induced
p,p'-DDD	0.040	ND	0.050	-	-	Not induced
Dipentyl phthalate	0.035	-	0.010	-	-	Not induced
p,p'-DDE	0.034	ND	0.012	ND	-	53.6 μg/L
Dipropyl phthalate	0.024	-	0.0018	-	-	Not induced
Di-*n*-butyl phthalate	0.023	ND	0.0063	-	-	Not induced
Dihexyl phthalate	0.023	-	0.013	-	-	Not induced
Octachlorostyrene	0.023	-	0.021	-	-	Not induced

Benzophenone	0.021	-	ND	-	-	501 μg/L
Beta-Hexachlorocyclohexane	0.020	-	0.0016	ND	-	Not induced
Di-(2-ethylhexyl) adipate	0.014	-	0.041	-	-	Not induced
Diethyl phthalate	0.012	-	-	ND	-	Not induced
2,4-Dichlorophenol	0.0037	-	0.0021	ND	-	324 μg/L
Amitrole	-	-	-	-	-	Not induced
Hexachlorobenzene	-	-	-	-	-	Not induced
4-Nitrotoluene	-	-	-	-	-	Not induced
Pentachlorophenol	-	-	-	-	-	Not induced

As for positive controls, 17β-estradiol and DHT were used for medaka estrogen and androgen assays, respectively. Data are shown as relative EC_{50} values (%) compared to the positive controls (100%). Weak positive, but EC_{50} was not detected (ND). No activity (-). Chemicals were in the order of the strength of ERα binding activity.