



**ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

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**Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA) of the
Test Guidelines Programme**

THE DRAFT DRP ON FISH RECEPTORS AND IN VITRO ASSAYS

First Meeting of the Advisory Group on Endocrine Disrupter Testing and Assessment (EDTA AG)

17th-18th May 2010, OECD Headquarters, Paris, France

Patric Amcoff

Tel (33) 1 45 24 16 19; Fax (33) 1 44 30 61 80; email: patric.amcoff@oecd.org

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The draft DRP on fish receptors and *in vitro* assays

WNT22 agreed to the proposal from the Secretariat to try to finalise the draft DRP on Fish Receptors as soon as possible after the WNT meeting. The Secretariat has compiled the comments after the last WNT commenting round and has consulted with the *ad hoc* Fish Receptor DRP Expert Group and amended the draft DRP accordingly. If approved by the EDTA AG, the draft DRP will be submitted to the WNT for approval by written procedure and subsequently sent to the Joint Meeting for declassification.

ACTION REQUIRED: ***The EDTA Advisory Group is invited to***
 (i) approve the draft DRP, as appropriate
 (ii) discuss and agree on the 4 recommendations of the DRP

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No. XX

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

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**OECD Environment Directorate,
Environment, Health and Safety Division**

**2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

PREAMBLE

1. At the 2nd meeting of the Validation Management Group for Non-Animal testing (VMG NA) in 2004, the question was raised by ICAPO (International Council for the Protection of Animals in OECD Programmes) to prepare a Detailed Review Paper (DRP) on “*In Vitro* Vitellogenin Assays”. The assays covered by the DRP were intended for the level 2 of the Conceptual Framework of the Endocrine Disrupters Testing and Assessment Task force (EDTA), “*In Vitro* Assays Providing Mechanistic Data”.

2. 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 3rd 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.

3. A first draft was presented to the WNT 18 in 2006 and since there were some issues raised on the scope of the DRP, the WNT18 agreed to ~~narrow it down to address~~ focus the DRP more on fish *in vitro* estrogen and androgen receptor binding and transcriptional activation assays. A new SPSF was submitted from the lead countries Japan, Sweden and the UK.

4. A redrafted 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 5 meeting further edited the draft before being re-submitted to VMG eco and then further edited by the Secretariat.

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
ac:	Arctic charr
acSBP:	Arctic charr Sex Steroid-Binding Protein
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- <i>n</i> -Buthylhexyl Phthalate
DBP:	Di- <i>n</i> -Butyl Phthalate
DDA:	Bis(<i>p</i> -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:	17 β -Estradiol
E3:	Estriol
EC₅₀:	Effective concentration for 50% maximal response
EDSP:	Endocrine Disruption Screening Program
EDTA:	Endocrine Disrupters Testing and Assessment
EE2:	17 α -Ethinylestradiol
EPC:	<i>Epithelioma Papulosum Cyprini</i>
ER:	Estrogen Receptor
ERE:	Estrogen Responsive Elements
ESTs:	Expressed Sequence Tags
fhERα:	<i>Fundulus heteroclitus</i> 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 <i>S</i> -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
IC₅₀:	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- <i>t</i> -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:	Rainbout trout
rbtAR :	Rainbout trout Androgen Receptor
rbtER :	Rainbout trout Estrogen Receptor
qRT-PCR:	quantitative Reverse Transcription Polymerase Chain Reaction
SBP :	Sex Steroid-Binding Protein
SHBG:	Sex Hormone-Binding Globulin
ss :	Spotted seatrout
ssER:	Spotted seatrout Estrogen Receptor
T :	Testosterone
TA :	Transcriptional Activation Assays
TAM:	Tamoxifen

TB:	Trenbolone <u>Trenbolone</u>	
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:	<i>Zona Radiata</i>	

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 amphibians, reptiles, birds, or the invertebrates. The assays covered by the |
DRP are for the level 2 of the Conceptual Framework of the Endocrine Disrupters Testing and Assessment Task force (EDTA), “*In Vitro Assays Providing Mechanistic Data*”.

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 (uterotropic) or the Uterotropic 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 17 β -estradiol (E2), but may still produce detrimental biological effects. (Barnes *et al.*, 2008; Focazio *et al.*, 2008). Induced hormone levels are routinely measured using commercial testing kits, ~~though these fail to relate to actual effects.~~ 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.~~ 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 and TA assays for human ER α , AR assays and a H295R Sterodogenesis assay. The Test Guideline 440 for the Uterotrophic Bioassay, a screening assay for estrogenic effects in rodents, was adopted in 2008- (OECD 2008a). The Test Guidelines for the Hershberger assay, (OECD 2009b), the updated Test Guideline 407 (Repeated dose 28-day oral toxicity) (OECD 2008b) and the Test Guideline for the 21-day Fish Screening Assay ~~are expected to be~~ (OECD 2009c) have all been approved in 2009. 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 laevis* 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 ~~high priority~~ 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* estrogen receptor (ER) and androgen receptor (AR) binding and/or gene expression, an *in vitro* assessment of steroidogenesis and mammalian (rat) and non mammalian *in vivo* assays. *In vivo*, the uterotrophic assay detects estrogens, while steroidogenesis, anti-thyroid activity, anti-estrogenicity and HPG function are assessed in a “Pubertal Female Assay”. (Anti-)androgens are detected in the Hershberger Assay (weight of AR-dependent tissues in castrate-immature-male rats) and (anti)androgens and chemicals affecting steroidogenesis and thyroid hormone activity are assessed in the pubertal male. Fish and amphibian assays have also been developed and validated.~~ 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 uterotrophic assay for estrogen agonism, 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 tropicalis* 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).

II. DESCRIPTION OF METHODS

Introduction

11. Molecular techniques can be potentially applied in assessing the adverse biological reproductive effects of endocrine-disrupting chemicals in aquatic organisms. Fish ER, AR and progesterone receptor (PR), ~~amphibian thyroid hormone receptor (TR)~~ and invertebrate ecdysone receptor, VTG, steroid biosynthesis enzymes (aromatase, sulfotransferase and hydroxysteroid dehydrogenase) are potentially useful for assessing such chemicals. Molecular methods of gauging VTG and *zona radiata* (ZR) expression and protein concentrations have included immunoassay and quantitative reverse transcription polymerase chain reaction (qRT-PCR). The isolation of key gene expression products (*e.g.*, aromatase, ZR, VTG) from a wider range of fish species using degenerate primers is essential. Endocrine disruption in invertebrates has received less attention compared with fish, partly because ~~the~~ 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 cross-species PCR-based assays, or even microarray ~~systems~~ 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 ~~among~~ 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 rbtER α and human ER α (hER α). The E2 concentration required to achieve maximal binding in a yeast expressed ~~receptors~~ receptor (Matthews *et al.*, 2000) was 10-fold higher for the rbtER than that for other ~~species~~ species' ERs, including hER. In rbtER α , the 50% maximal effective concentration (EC₅₀) 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~~ 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. ~~Amphibian metamorphosis is marked by dramatic, thyroid hormone-induced changes involving gene regulation by TRs. Amphibian metamorphosis represents a promising model for the identification of thyroid system disrupting chemicals due to the pivotal role played by thyroid hormones for the initiation and regulation of metamorphosis (Opitz *et al.*, 2005; Tietge *et al.*, 2005; Degitz *et al.*, 2005; Mitsui *et al.*, 2006). An important aspect of bioassay development is the identification and evaluation of sensitive and diagnostic endpoints, which may include TR-binding~~

~~and transactivation assays.~~

1413. This DRP summarizes the use of receptor binding assays using extracted ~~receptor~~ER and AR proteins and recombinant receptors as a means of primary screening of environmental samples and chemicals for estrogenicity, anti-estrogenicity, androgenicity, and anti-androgenicity in fish species, which avoids species and seasonal variation in receptor response to ligand binding. Transcriptional ~~Activation~~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

15.14. 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 *Escherichia 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, [³H]-E2, for binding to the ER. In a typical competitive hormone binding assay, ~~a high speed centrifugal fraction of cytosol, cell extract or recombinant ER LBD fusion protein prepared using *E. coli*~~ is incubated with [³H]-E2 and various concentrations of unlabelled test compounds. If compounds compete with the [³H]-E2 for receptor binding, they will displace a fraction of the [³H]-E2 from the receptor in a concentration dependent manner. As the concentration of the unlabelled competitor increases, more [³H]-E2 is displaced from the ER. Usually, the free [³H]-E2 is separated from the bound [³H]-E2 by dextran-coated charcoal or hydroxyapatite treatments or other methods and receptor-bound [³H]-E2 is quantified by liquid scintillation counting. Non-specific binding is measured by addition of excesses of ~~radioinert~~radio-inert diethylstilbestrol (DES) or E2. The specific binding of [³H]-E2 to the ER is calculated by subtracting the amount of non-specific binding from the amount of [³H]-E2 bound in the absence of a competitor. Decreased specific binding of the [³H]-E2 in the presence of a test sample suggests that the sample contains ~~compounds that~~ 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)

16.15. 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.

17.16. 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. ~~Thus, while the background level of VTG is low, the production of VTG mRNA or protein is easily measured in these systems as a response to ER agonism.~~ 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.

1817. 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, resulting and results in the production of an easily detected product.

1918. 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 EC₅₀ for the test substance can be determined and compared with that for the reference estrogen.

2019. 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.

2120. Another promising assay system for ER TA has been recently 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) ER α , 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 jgsER α . ~~This indicates~~ The latter thus indicates that the GAL4 DNA-binding domain fused with jgsER α 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 jgsER α . 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. ~~The new system appears to be more suitable than the ERE-luciferase system for assays of ER transactivation~~ (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 superfamily 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 ER α , ER β 1 and ER β 2-forms but the teleost ER β 2-form appears to be closely related to the teleost ER β 1, 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 ER α and ER β 1 (Thornton, 2001). Indeed, these two forms of ER have been previously found in mammals, fish, birds, reptiles and amphibians. cDNAs encoding for ER α 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 (rft) 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 [³H]-E2 to the rftER. 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 [³H]-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 [³H]-E2.

23. Relative binding affinities of endocrine active compounds to rftER 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 rftER (0.8-2.7 nM); however, in both species, females and males had similar K_d (fhmER, 8.6 vs. 6.2 nM; rftER, 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 rftER 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), testosterone (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. [3H] 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. Rainbout trout ER from liver tissue competitive binding assays with 16 chemicals (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% for 13 chemicals and partial or no binding for 3 additional chemicals. Eleven lower to no affinity chemicals (RBA < 0.1%) were further 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 tested that (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). The monohydroxy metabolite of MXC was the only chemical tested that apparently bound rbtER but did not induce VTG mRNA. Data were presented illustrating the utility of the combination of the two assays for interpreting the role of metabolism in VTG induction, as well as the sensitivity of the assays for measuring enantiomer selective binding and ER-mediated induction. The combined approach appears particularly useful in interpreting the potential relevance of extremely low affinity chemical binding to fish receptors (RBA = 0.01-0.0001%) within a defined toxicity pathway as a basis for prioritizing within large chemical inventories of environmental concern (Schmieder *et al.*, 2004). 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 phthalate, 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 $10^4 - 2 \times 10^5$ times less potent than E2 in displacing specifically bound [^3H]-E2. ~~The displacement of bound [^3H]-E2 did not increase when these compounds were tested in combination.~~ 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 [^3H]-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 [^3H]-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-hydroxytamoxifen (4-HT), EE2 > GEN, ZEA > OP, NP, and *o,p'*-dichloro-diphenyl-trichloroethane (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. ~~The~~ A study investigated the ability of 34 natural and synthetic chemicals to compete with [^3H]-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 hER α (GST-hER α def), mouse ER α (GST-mER α def), 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 [^3H]-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~~. 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. ~~Rainbow trout ER from liver tissue competitive binding assays for 16 chemicals yielded calculable RBA from 179 to 0.0006% for 13 chemicals and partial or no binding for an additional 3 chemicals. Eleven lower to no affinity chemicals (RBA < 0.1%) were further 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. Data were presented illustrating the utility of the two assays in combination for interpreting the role of metabolism in VTG induction, as well as the sensitivity of the assays for measuring enantiomer selective binding and ER-mediated induction. The combined approach appears particularly useful in interpreting the potential relevance of extremely low affinity chemical binding to fish receptors (RBA = 0.01–0.0001%) within a defined toxicity pathway as a basis for prioritizing within large chemical inventories of environmental concern (Schmieder *et al.*, 2004).~~ (Table 1 Relative affinity of chlordane and *o,p'*-DDT or DDE for rbtER was studied. Chlordane 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 and VTG induction assay results indicated that chlordane, *o,p'*-DDT and *o,p'*-DDE were weakly estrogenic in juvenile trout (Donohoe and Curtis, 1996).

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 rbtER α 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 (EC₅₀). 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 EC₅₀ for estrogen-responsive luciferase activity was 3.8 x 10⁻⁹ M at 11 °C and 7.4 x 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 EC₅₀ of the weak ER agonist 4-tert-pentylphenol was 7.6 x 10⁻⁷ M at 11 °C and 6.9 x 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 is could be an important endocrine disrupter through a direct effect on ER transcriptional activity and may affect a number of estrogen signaling pathways.

Spotted seatrout (*Cynoscion nebulosus*)

Binding – endogenous receptors

34. Spotted seatrout (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 seatrout. 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 seatrout 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*, ER α (fhER α) was cloned and the ER α binding assay was carried out (Urushitani *et al.*, 2003) (Table 1). Recombinant fhER α LBD fusion protein was prepared using *E. coli*. fhER α binding affinities to various concentrations (1×10^{-4} – 1×10^{-11} M) of OP, NP, BP, di-*n*-butylhexyl phthalate (DBHP), octachlorostyrene (OCS) and tributyltin chloride (TBT) were measured. E₂ bound to fhER α with an IC₅₀ value of 5.5×10^{-9} M. The RBA values of the other chemicals were calculated as a percent ratio of the IC₅₀ values of test substances relative to E₂. RBA of OP and NP for fhER α 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 fhER α . The binding potency of OCS to fhER α was estimated as 0.02% of E₂.

Medaka (*Oryzias latipes*)

Binding – recombinant receptors

37. Medaka (m) ER α and mER β 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 mER α 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-ethylhexyl adipate showed weak binding affinities (0.012-0.045%). RBA of OP, NP and DEHP for mER β were 0.8-0.83%. Phthalates showed weak binding affinities (0.002-0.006% of E2).

~~38. *In vitro* competitive enzyme immunoassay for ERs using ER-LBD proteins from human, *Xenopus*, Japanese quail and medaka were established and compared the species specificity of the ability of NP and OP to bind ERs. Although a significant difference was not detected among ER β of human, quail and medaka, NP and OP exhibited the higher affinity for the mER α than hER α . These results indicate the species specificity of the capacity of chemicals to bind ERs (Nishizuka *et al.*, 2004).~~

Transcriptional Activation – engineered systems

~~3938.~~ 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 E₂ for mER α were 1.3 and 0.35%, respectively. Other chemicals including phthalates and TBT were negative. For mER β and mAR reporter gene assay (value obtained for 5 α -dihydrotestosterone (DHT) was 100%), all the chemicals studied, including phthalates, alkylphenols, TBT and OCS were negative (Table 3).

Transgenic animal system

~~4039.~~ 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 harboring 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.

4140. 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

4241. 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 fhmER α at an elevated temperature (37°C) compared to 23°C.

Zebrafish (*Danio rerio*)

Transcriptional Activation – engineered systems

4342. Three functional ER forms have been cloned and characterized in zebrafish (zf), zfER α , zfER β 1 and zfER β 2 (Menuet *et al.*, 2002) (Table 2). Identity was 40.5% between zfER α and zfER β 2, and 51.5% between zfER β 1 and zfER β 2, 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 zfER β 2 and 0.75 nM for both zfER α and zfER β 1, indicating that zfER β 2 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 zfER β 2 was slightly more sensitive than zfER α and zfER β 1. ICI164384 or 4-hydroxytamoxifen~~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.

44. Functional *in vitro* and *in vivo* reporter gene assays were developed for the rapid determination of exposure to (xeno)estrogens. The *in vitro* ER-mediated Chemically Activated LUciferase gene eXpression (ER-CALUX) assay used T47D human breast cancer cells stably transfected with an ER-mediated luciferase gene construct. In the *in vivo* assay, transgenic zebrafish were used in which the same luciferase construct has been stably introduced (Murk et al., 1996). In both assays, luciferase reporter gene activity can be easily quantified following short-term exposure to chemicals activating endogenous ERs. Sensitivity of these assays was compared using known (xeno)estrogenic compounds (Legler et al., 2002). Exposure to the 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, whereas in the *in vitro* ER-CALUX assay, EE2 and E2 were equipotent. Although the xenoestrogens *o,p'*-DDT and NP were full estrogen agonists in the *in vitro* ER-CALUX assay, only *o,p'*-DDT demonstrated weak dose-related estrogenic activity *in vivo*. Using transiently transfected recombinant ER and reporter gene constructs, EE2 also showed relatively potent activation of zfER α and zfER β compared to hER α and hER β . The zfER β and zfER γ showed higher transactivation by (xeno)estrogens relative to E2 than hER β .

Transgenic animal system

4543. 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, they tend to bioaccumulate in aquatic biota. In surface waters, concentrations between 1 ng/L and 5 μ g/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 hER α and hER β 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 zfER β , β and was more pronounced on zfER γ .

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 LUciferase 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 zfER α and zfER β compared to hER α and hER β . The zfER β and zfER γ showed higher transactivation by (xeno)estrogens relative to E2 than hER β .

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 μ M 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

4647. 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 IC₅₀ 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 IC₅₀ 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

47. — Recombinant ccER α and ccER β were obtained after transient transfection of COS-7 cells with ccER expression plasmids. Plasma from adult female channel catfish was the source of estrogen-binding proteins (Sex Hormone-Binding Globulin, SHBG). [³H]-E2 was used in standard radioligand-binding assays to characterize the binding properties of ccSHBG and to estimate the inhibition constants for various estrogenic compounds. Binding of [³H]-E2 to ccSHBG was saturable and of high affinity with *Kd*=1.9 nM and *B_{max}*=14.3 pmol/mg protein. Additionally, ccSHBG displayed binding specificity for androgens and estrogens. Endosulfan, NP, and OP displaced [³H]-E2 binding to ccSHBG albeit only at very high concentrations, whereas dieldrin and atrazine showed little displacement activity even at the highest concentrations used. EE2 had

higher affinity than E2 for eeSHBG. This finding differs from results with human and rainbow trout SHBG. The alkylphenolic compounds (NP and OP) displayed some ability to displace [³H]-E2 from eeER α and eeER β . Recombinant ER α and ER β were obtained after transient transfection of COS7 cells with ER expression plasmids. NP and OP displayed some ability to displace [³H] E2 from ER α and ER β at high concentrations, but dieldrin and atrazine had little binding activity for both ER subtypes and endosulfan for eeER β . The xenobiotics tested generally showed equivalent or greater affinity for ER α than ER β , whereas endogenous estrogens, such as E2, E1 and E3, had much greater affinity for ER β than ER α . These observations suggest that results of studies using fish tissue-ER extracts should be interpreted with caution, since both ER subtypes may be present, and that the binding of xenoestrogens to SHBG should be taken into account for proper assessment of endocrine disruption caused by environmental contaminants (Gale *et al.*, 2004) (Table 1).

Atlantic croaker (*Micropogonias undulatus*)

Binding – endogenous receptors

4849. 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 ($K_d=0.40$ nM in cytosolic extracts and $K_d=0.33$ 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 [³H]-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

4950. 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 [³H]-E2 with high affinity and low capacity ($K_d = 2.5-4.4$ nM and $B_{max} = 27-97$ fmol/mg protein). Atlantic salmon ER exhibit similar [³H]-E2 binding

characteristics, although livers from female fish contained a 2-3 times higher amounts of ER than the males. In competition studies with [³H]-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-hydroxytamoxifen, 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.

Arctic charr (*Salvelinus alpinus* L.)

Sex Steroid-Binding Protein (SBP)

50. — A specific sex steroid binding protein (SBP) is believed to be involved in regulation of circulating sex steroids, steroid delivery to target cells and intracellular signaling in sex steroid-sensitive tissues. Interactions between xenoestrogens and the plasma SBP in Arctic charr (ac) were determined using ligand protein binding studies (Tollefsen *et al.*, 2004). The test compounds were all able to displace [³H]-E2 from the acSBP in a competitive and dose-dependent manner. The acSBP affinities for the xenoestrogens ranged over several orders of magnitude (E2 >> EE2 > ZEA > DES > GEN > BPA, OP >>> *o,p'*-DDT, and dieldrin), but were consistently lower than that of E2 (about 4×10^2 – 10^6 fold less potent). The RBA for selected chemicals were independent of gender, age and maturation status, as well as variations of acSBP binding affinity. The affinity of endogenous steroids and estrogen mimics for the acSBP shows a high correlation to the affinity for the rbtSBP, thus suggesting a phylogenetically conserved ligand binding site between closely related species. Furthermore, it is argued that interaction with the SBP-mediated processes may introduce novel pathways for endocrine disruption, which may work in concert with the classical receptor-mediated effects.

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 [³H]-E2 from the hepatic ER of carp. The affinity ranking for ligands on the basis of statistical differences of the IC₅₀ values were E2 > TAM > BPA > OP > 3,3,4',4-tetrachlorobiphenyl > diethylphthalate > 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 ER α 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 ER α (1680 bp) and ER β (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 EC₅₀ values for the compounds tested showed that ER β was 3-21-fold more sensitive to natural steroidal estrogens and 54-fold more sensitive to EE2 as compared to ER α . 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. β 1), 43% (α vs. β 2), and 52% (β 1 vs. β 2). 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 ER β of human, quail and medaka, NP and OP exhibited the higher affinity for the mER α than hER α . 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 fhmER α was higher, than that of hER α (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. ~~Only limited~~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). ~~Therefore, species differences and strain differences in sensitivities for chemicals need to be studied in fish. 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) for review).

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 evolutionary biology. The androgen receptor (AR) belongs to the nuclear receptor (NR) superfamily and is the key molecule controlling the expression of such masculine phenotypes. In teleost fishes, two distinct paralogous copies of ARs 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, an 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 acivities 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

5859. Two AR cDNA clones (rbtAR α and rbtAR β) from the rainbow trout testis were isolated (Takeo and Yamashita, 1999) (Tables 1 and 2). To investigate the functions of the rbtAR α , the ligand binding ability and transcriptional activity of rbtAR α were analyzed (Takeo and Yamashita, 2000). In ligand-competition experiments, T (IC₅₀: 3 x 10⁻⁹ M) competed with [³H]-mibolerone binding for rbtAR α 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 (EC₅₀: 5 x 10⁻⁹ M) and 11-KT (EC₅₀: 6 x 10⁻⁹ M) showed similar efficiency upon co-transfection into *epithelioma papulosum cyprini* (EPC) cells with a rbtAR α expression vector and an androgen-responsive element-based reporter gene. These results indicate that activation of rbtAR α 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 [³H]R1881, a high-affinity synthetic androgen, revealed an equilibrium dissociation constant (Kd) 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

5961. Both AR and ER α 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 *K_d* 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 17 α - and 17 β -trenbolone (TB); AR antagonists such as *p,p'*-DDE, linuron, and vinclozolin; and E2.

6062. 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 masculinization (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

6163. Female mosquitofish (*Gambusia affinis holbrooki*) downstream from the Kraft paper mills in Florida display masculinization 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. ~~Water samples were tested for androgenic activity from a reference site upstream of a Kraft pulp and paper mill on the Fenholloway River, from 3 sites downstream from the mill, and from another reference site on the Econfina River in Florida, where there is no paper mill. Anal fin ray morphology was examined in mosquitofish from these rivers for evidence of masculinization (Parks *et al.*, 2001).~~ Eighty percent of the female mosquitofish from the

Fenholloway River were partially masculinized while another 10% were completely masculinized, 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 μ M), 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-translocation assay. PME bound hAR and induced translocation 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 masculinization of female mosquitofish.

Transcriptional Activation – engineered systems

6264. 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*) were examined. Two forms of mosquitofish AR, mqfAR α and mqfAR β , were cloned (Sone *et al.*, 2005). The mRNA expression levels of mqfAR α and mqfAR β were transiently increased in the anal fin of adult females at day 3 following exposure to TB (1–10 μ g/L) or MT (0.1–10 μ g/L), a pharmaceutical androgen used as a positive control. Gonopodium differentiation from the adult female anal fin was induced after 28 days of exposure to TB (1–10 μ g/L) or MT (0.1–10 μ g/L). Gonopodium differentiation also was induced in all mosquitofish fry exposed for 28 days to 0.3, 1 or 10 μ g/L TB. Furthermore, spermatozoa were observed histologically in the testes of male fry exposed for 28 days to 1 or 10 μ g/L TB; spermatozoa are normally observed only in the testes of mature males. Surprisingly, all female fry exposed for 28 days to 1 or 10 μ g/L TB displayed the formation of an ovotestis, as spermatozoa were found in the ovary. Thus, TB, like MT, induced masculinization of the anal fin accompanied by a transient up-regulation of mqfAR α and β in adult females. TB also and two forms of mosquitofish AR, mqfAR α and mqfAR β , 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

6365. 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; Koplín *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 recombinant 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.

6466. 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).

6567. In hER, Kinnberg (2003) compared sensitivities among the binding assay, the cell proliferation assay using MCF-7 mammary tumor 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 ~~assay~~ 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 economical/cheap *in vitro* assays systems using fish receptors needs to be evaluated, and if possible developed, validated and turned into OECD Test Guidelines.

6668. 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 metabolism/metabolization and thus longer systemic half-life. A 7-year, whole lake experiment at the Experimental Lakes Area in northwestern 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 their mimics/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.

6769. 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 metabolism/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 EC₅₀ values. Xenobiotic metabolism which 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 of this type is needed on more/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 ER α and ER β and the significant contribution of ER β 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

6871. 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.

6973. In vitro assayassays applied to chemical-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, prprevious 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: Since there doesn'tThere seem to be be any available test systems for certain potential advantages by using sliced fish livers and primary hepatocyte cultures or liver slice assays, however, since there are no available standardised test systems that have been evaluated or validated for broad-regulatory applicability purposes, it is recommended as a first step that the VMG eco and VMG NA establish an expert group to further disseminate in detail existing assays and available data be further disseminated for possible further additional recommendations towards validation studies, retrospective performance assessments or whether a draft Test Guideline can be developed. Primarily assays using larger fish, e.g., rainbow trout, should be considered since this has the potential of reducing animal usage. 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.

70 **Yeast-Based Assays with Fish Steroid Hormone Receptors**

74. By comparing sensitivities of a number of assays, the yeast-based YES-assay has and YAS (for AR) -assays have been recommended to for use for in monitoring estrogenic and androgenic activity in contaminated environmental samples such as influent sewage water containing toxic compounds (Kinnberg, 2003) (see paragraph 65)-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-assay is therefore/YAS-assays are recommended for further detailed evaluation by the VMG-eco and VMG-NA by the establishment of an expert group that will further investigate available YES-system evaluations primarily focusing on assays with fish steroid hormone receptors and report its progress. 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 the VMGs for certain compounds or chemical classes or other potential limitations, need to be clearly demonstrated before any further discussion. 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.

71. For receptor binding assays, recombinant proteins or extracted proteins from tissues can be used. Gene activation 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: hER α , quail ER α (qER α), alligator ER α (aER α), salamander ER α (sER α), and fathead minnow (fhmER α). Saturation binding analyses indicated E2 dissociation constants (Kd) as: 0.22 nM for hER α , 0.28 nM for sER α , 0.44 nM for aER α , 0.58 nM for qER α , and 0.58 nM for fhmER α . 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 hER α to 1.01 nM with sER α for E2 and from 0.68 nM with sER α to 1.20 nM with qER α for EE2. DHT was a weak binder with IC50's ranging from 3.3 μ M with hER α to 39 μ M with fhmER α , and C did not bind to any of the receptors at concentrations up to 100 μ M. 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 suggested suggests evidence of species differences in affinity and sensitivity of hormone receptors.

Recommendation 3: Receptor binding and gene activation TA assays using ER, AR

~~and TR-AR from various fish species is needed and a study should be designed and conducted to directly compare the compared in terms of their sensitivity, specificity and practicality of considering different approaches. 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 for screening and prioritization leading to further testing, more information will become available to analyze for analysing the relationships between the in vitro and the in vivo assays for intra-, and inter-species comparisons. The and their potential regulatory utility. A majority of these will assays are likely to be assays that parallel mammalian-based in vitro assays. In addition, and the ER-mediated production of VTG naturally occurring in fish livers has allowed the development of endogenous gene activation assays that are not available need for mammalian systems. Future validation studies of fish specific fish-receptor assays should ideally use chemicals based on results from existing validated in vivo tests. be demonstrated prior to any development of Test Guidelines.~~

72 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 ~~gene activation~~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 ~~gene activation~~TA assays compare the response of the test chemical to that of a positive control, e.g., E2 for ER binding and ER-mediated ~~gene activation~~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 ~~chemical~~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 species. Future validation studies of fish receptor assays should ideally use chemicals based on results from existing validated OECD Test Guidelines.

~~73. The current DRP focuses on in vitro assays available for fish species and does not attempt to include additional wildlife taxa such as amphibians, reptiles, birds, or the invertebrates.~~

~~**Recommendation 5:** If it is later determined that steroid hormone receptor binding and gene activation assays are needed for additional wildlife species as more information becomes available, assays for species with available in vivo toxicity testing data would be particularly useful, including *Xenopus laevis*, *Xenopus (Silurana) tropicalis* and Japanese quail. These could include TR assays for *X. laevis* and *X. (S.)tropicalis*, and binding assay using transthyretin from amphibian and avian species. Additionally, assays for invertebrate arthropods (e.g., amphipods, copepods, *Daphnia magna*, mysids) and validation of an in vitro assay for the ecdysone receptor may also be needed. Potential suitable models exist based on the published literature (Dinan, 1985; Dinan et al., 2001; Grebe et al., 2004), and should be explored further.~~

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Table 1. Receptor Binding Assays

Type/isoform	Source	Tissue	Species	Chemicals	REFS	paragraph		
				classes tested	No. of chem tested			
ER	1) Endogenous							
mixed		liver	a) cytosol	rainbow trout	phthalates, benzophenones, subst benzenes BHA, subst.phenols benzoic acids steroids	20	Jobling <i>et al.</i> , 1995	22
mixed		liver	a) cytosol	rainbow trout	alkylphenols phytoestrogen steroids	11	Denny <i>et al.</i> , 2005	23
mixed		liver	a) cytosol	rainbow trout	pharmaceuticals pesticides alkylphenols alkylphenols	16	Schmieder <i>et al.</i> , 2004	24
mixed		liver	a) cytosol	rainbow trout	phthalates pesticides	18	Knudsen and Pottinger, 1999	25
mixed		liver	a) cytosol	rainbow trout	phytoestrogen phytoestrogen steroids	3	Arukwe <i>et al.</i> , 1999	26
mixed		liver	a) cytosol	rainbow trout	pharmaceuticals phytotoxins/mycotoxins pesticides alkylphenols	15	Tollefsen <i>et al.</i> , 2002	27
mixed			a) cytosol	rainbow trout	Chlordecone, <i>o,p'</i> -DDT, <i>o,p'</i> - DDE, <i>p,p'</i> -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 alkylphenol DDT, DDEs	13	Nimrod and Benson, 1997	46

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 <i>et al.</i> , 2002	49	
mixed		liver	a) cytosol	carp	steroids pharmaceuticals Industrial chemicals pesticides	22	Kloas <i>et al.</i> , 2000	51	
<hr/>									
	2)Recombinant Expression cells	receptor details		ER species origin					
ER alpha	E. coli	LBD		rainbow trout	steroids alkylphenols mycotoxins pesticides	34	Matthews <i>et al.</i> , 2000	28	
ER alpha	E. coli	full length		rainbow trout	phytoestrogens heavy metals alkylphenols	6	Guevel <i>et al.</i> , 2000	33	
ER alpha	yeast cells	LBD		mummichog	phthalates styrene TBT	8	Urushitani <i>et al.</i> , 2003	36	
ER alpha beta	E. coli	LBD		medaka	alkylphenols phthalates styrene TBT	13	Nakai <i>et al.</i> , 2003	37	
ER alpha beta	E. coli	LBD		medaka	pharmaceuticals alkylphenols	3	Nishizuka <i>et al.</i> , 2004	38	
ER alpha beta	COS-7 cell	full		channel catfish	steroids alkylphenols dieldrin	9	Gale <i>et al.</i> , 2004	47	

ER alpha	E. coli	LBD		red sea bream	endosulfan alkyl phenols	3	Nakai <i>et al.</i> , 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 <i>et al.</i> , 2004b	59
AR alpha	COS-1	full		fathead minnow	flutamides	2	Ankley <i>et al.</i> , 2004	60
TR	1) Endogenous							
	2) cytosol	liver	a) cytosol	rainbow trout	phthalates	5	Knudsen and Pottinger, 1999	25
steroid-binding protein	1) Endogenous							
		serum		eatfish	steroids alkylphenols endosulfan	7	Gale <i>et al.</i> , 2004	47
		serum		atlantic charr	steroids pharmaceuticals phytotoxins/mycotoxins pesticides	9	Tollefsen <i>et al.</i> , 2004	50
	serum		carp	alkylphenols steroids pharmaceuticals	13	Kloas <i>et al.</i> , 2000	51	

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	alkylphenols DDTs	12	Ackermann <i>et al.</i> , 2002	31
ER alpha	RTH-149	a) full length	a) luciferase	rainbow trout	EE2 penthylphenol alkylphenols	2	Hornung <i>et al.</i> , 2003	temp sens of rbtER 32
ER alpha beta	HeLa	a) full length	a) luciferase	medaka	phthalates TBT OCS	13	Nakai <i>et al.</i> , 2003	37
ER alpha beta1 beta2	CHO	a) full length	a) luciferase	zebrafish	steroids pharmaceuticals	10	Menuet <i>et al.</i> , 2002	43
ER alpha beta gamma	HEK293	a) full length	a) luciferase	zebrafish	steroids DDTs nonylphenol	6	Legler <i>et al.</i> , 2002	44
ER alpha beta	CHO-K1	a) full length	a) luciferase	roach	steroids pharmaceuticals	6	Katsu <i>et al.</i> , 2007a	53

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ER alpha beta	HEK293	a) full length	a) luciferase	mosquitofish	alkylphenols steroids	4	Katsu <i>et al.</i> , 2007b	54
ER alpha	HeLa BF-2	a) full length	a) luciferase	rainbow trout	steroids pharmaceutica ls alkylphenols phytoestrogen s DDT	10	Sumida <i>et al.</i> , 2003	56
AR	recombina nt CELL TYPE	receptor details						
	HeLa	a) full length	a) luciferase	medaka	alkylphenols phthalates TBT OCS	13	Nakai <i>et al.</i> , 2003	37
AR alpha beta	HEPG2	a) full length	a) luciferase	mosquitofish	steroids	6	Katsu <i>et al.</i> , 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	
17 β -Estradiol	100	100	100	100		21.9 ng/L
Dihydrotestosterone					100	
Ethinylestradiol	78	97	80	226	5.9	14.8 ng/L
4- <i>t</i> -Octylphenol	16	1.3	0.83	-	-	64.1 μ g/L
4-Nonylphenol (branched)	8.1	0.35	0.83	-	-	22.5 μ g/L
Di-(2-ethylhexyl) phthalate	0.79	-	0.37	-	-	Not induced
<i>trans</i> -Nanochlor	0.60	ND	0.022	-	-	Not induced
<i>o,p'</i> -DDT	0.54	ND	0.17	-	-	1.5 μ g/L
Bisphenol A	0.48	0.076	0.31	-	-	334 μ g/L
<i>cis</i> -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
<i>p,p'</i> -DDT	0.12	ND	0.069	-	-	Not induced
Dicyclohexyl phthalate	0.045	-	0.016	-	-	Not induced
<i>p,p'</i> -DDD	0.040	ND	0.050	-	-	Not induced
Dipentyl phthalate	0.035	-	0.010	-	-	Not induced
<i>p,p'</i> -DDE	0.034	ND	0.012	ND	-	53.6 μ g/L
Dipropyl phthalate	0.024	-	0.0018	-	-	Not induced
Di- <i>n</i> -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 μ g/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 μ g/L

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Amitrole	-	-	-	-	Not induced
Hexachlorobenzene	-	-	-	-	Not induced
4-Nitrotoluene	-	-	-	-	Not induced
Pentachlorophenol	-	-	-	-	Not induced

As the positive controls, 17 β -estradiol and DHT were used for medaka estrogen and androgen assays, respectively. Data are shown as relative EC₅₀ values (%) compared to the positive controls (100%). Weak positive, but EC₅₀ was not detected (ND). No activity (-). Chemicals were in the order of the strength of ER α binding activity.