Global Water Research Coalition

In Vitro Bioassays to Detect Estrogenic Activity in Enviromental Waters

LITERATURE REVIEW

Global Water
Research Coalition

IWA affiliate

IN VITRO BIOASSAYS TO DETECT ESTROGENIC ACTIVITY IN ENVIRONMENTAL WATERS

LITERATURE REVIEW

2006

Global Water Research Coalition: Global cooperation for the generation of water knowledge

GWRC is a non-profit organization that serves as the collaborative mechanism for water research. The product the GWRC offers its members is water research information and knowledge. The Coalition will focus on water supply and wastewater issues and renewable water resources: the urban water cycle.

The founder members of the GWRC are: the Awwa Research Foundation (US), CRC Water Quality and Treatment (Australia), EAWAG (Switzerland), Kiwa (Netherlands), Suez Environment- CIRSEE (France), Stowa - Foundation for Applied Water Research (Netherlands), DVGW – TZW Water Technology Center (Germany), UK Water Industry Research (UK), Veolia-Anjou Recherché (France), Water Environment Research Foundation (US), Water Research Commission (South Africa), Water Reuse Foundation and the Water Services Association of Australia.

These organizations are all in charge of a national research program addressing the different parts of the water cycle. They have provided the impetus, credibility, and initial funding for the GWRC. Each brings a unique set of skills and knowledge to the Coalition. Through its member organizations GWRC represents the interests and needs of 500 million consumers.

The Global Water Research Coalition is affiliated with the International Water Association (IWA). The GWRC was officially formed in April 2002 with the signing of the partnership agreement at the International Water Association 3rd World Water Congress in Melbourne. With the U.S. Environmental Protection Agency a partnership agreement was signed in July, 2003.

This study was jointly funded by GWRC members. GWRC and its members assume no responsibility for the content of the research study reported in this publication or for the opinion or statements of fact expressed in the report. The mention of trade names for commercial products does not represent or imply the approval or endorsement of GWRC and its members. This report is presented solely for informational purposes.

ACKNOWLEDGMENTS

Project Team
Heather Chapman, Ph.D.
Frederic Leusch, Ph.D.
CRC Water Quality and Treatment/Griffith University (Australia)

Lead Agent
Margaret Stewart
Water Environment Research Foundation (U.S.)

Project Steering Group
Seth Kullman, Ph.D.
Duke University (U.S.)

Deb Lester
King County (U.S.)

Elaine Francis, Ph.D.
U.S. Environmental Protection Agency (U.S.)

Djanette Khiari, Ph.D.
Awwa Research Foundation (AwwaRF) (U.S.)

Gordon Wheale
UK Water Industry Research (UK)

Issy Cafoor, Ph.D.
Yorkshire Water (UK)

Project Participants
Tiaan de Jager, Ph.D.
Natalie Aneck-Hahn, Ph.D.
Water Research Commission/University of Pretoria (South Africa)

Richard Lim, Ph.D.
Anne Colville, Ph.D.
University of Technology Sydney (Australia)

Louis Tremblay, Ph.D.
Landcare Research (New Zealand)

Leo Puijker, Ph.D.
Kiwa Water Research (The Netherlands)

Mike van den Heuvel, Ph.D.
University of Prince Edward Island (Canada)

Frank Sacher, Ph.D.
Technologiezentrum Wasser (Germany)

Nadine Dumoutier, Ph.D.
Jean-Michel Laine, Ph.D.
Suez Environment (France)

Global Water Research Coalition
Frans Shulting, Ph.D.
Global Water Research Coalition (UK/The Netherlands)

ABSTRACT

The presence of estrogenic (feminizing) compounds in drinking waters, source waters, and wastewater is of international concern because of potential adverse effects on exposed wildlife and humans. Chemical analysis has been problematic due to both the large number of compounds with estrogenic activity and the ultra-low concentrations that can cause estrogenic effects. Bioassays can integrate and measure the effects of complex mixtures and are becoming increasingly popular as screening tools. This project aims to validate a comprehensive battery (or "toolbox") of bioassays to detect estrogenic activity in a variety of environmental waters (such as recycled water) and provide a basis to assess the risk of exposure to biological organisms, including humans.

The purpose of this document is to provide an overview of all currently available bioassays to detect estrogenicity in environmental water samples. Each assay will be described and its advantages and limitations discussed to facilitate selection in the toolbox.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

ALP = Alkaline phosphatase
BG-1 = Human ovarian cancer cell line
CPRG = Chlorophenol red-β-D-galactopyranoside
E1 = Estrone
E2 = 17β-Estradiol
ED = Endocrine disruption
EDC = Endocrine-disrupting compound
EE2 = 17α-Ethynylestradiol
ELISA = Enzyme linked immunosorbent assay
ELRA = Enzyme-linked receptor assay
ER = Estrogen receptor
ERBA = Estrogen receptor binding assay
ER-CALUX = ER-mediated chemical-activated luciferase gene expression
ERE = Estrogen responsive element
ERLBD = ER ligand binding domain
FP = Fluorescence polarization
GAL4AD = GAL4 activation domain
GAL4DBD = GAL4 DNA binding domain
GFP = Green fluorescent protein
GSI = Gonadosomatic index
GWRC = Global Water Research Coalition
HAP = Hydroxyapatite
HELN = HeLa cells with ERE-Luc-Neo
HEP = Hepatocyte
HGELN = HeLa cells with Gal4-ER-Luc-Neo
MCF-7 = Human breast cancer cell line
MELN = MCF-7-ERE-βGlob-Luc-Neo
MVLN = MCF-7-Vit-Luc-Neo
OMPdecase = Orotidine-5'-phosphate decarboxylase
RTG-2 = Rainbow trout gonad cell line
STP = Sewage treatment plant
TMB = Tetramethylbenzidin
Vtg = Vitellogenin
yEFGP = yeast enhanced green fluorescent protein
YES = Yeast estrogen screen
Zrp = Zona radiata protein

The presence of estrogenic (feminizing) compounds in drinking waters, source waters, and wastewater is of international concern because of potential adverse effects on exposed wildlife and humans. Chemical analysis has been problematic due to both the large number of compounds with estrogenic activity and the ultra-low concentrations that can cause estrogenic effects. Bioassays can integrate and measure the effects of complex mixtures and are becoming increasingly popular as screening tools. This project aims to validate a comprehensive battery (or "toolbox") of bioassays to detect estrogenic activity in a variety of environmental waters (such as recycled water) and provide a basis to assess the risk of exposure to biological organisms, including humans.

The purpose of this document is to provide an overview of all currently available bioassays to detect estrogenicity in environmental water samples. Each assay will be described and its advantages and limitations discussed to facilitate selection in the toolbox.

1.0 Introduction

The endocrine system is composed of diverse glands which control hormone metabolism. Hormones in turn regulate a variety of biological functions including growth, metabolism, cell growth and proliferation, cell function and differentiation, sexual development and behaviour, and development of the immune system (Hadley, 1988). Most hormones bind to specific membrane receptors in target cells, triggering a cascade of cellular and biochemical events that eventually lead to gene transcription and *de novo* protein synthesis. Lipophilic hormones (such as steroid and thyroid hormones) pass readily through cell membranes and bind ligand-specific receptors, resulting in a receptor hormone complex. These complexes interact with specific transcription-control regions of nuclear DNA resulting in modulation of RNA and protein biosynthesis (Figure 1) (Hadley, 1988; Lodish et al., 1995; Zacharewski, 1997).

Steroid hormones (such as estrogens) can also initiate cellular events through a non-genomic mechanism (Metzger, 1995; Thomas, 2003). This action occurs via interaction with cell surface receptors (usually tyrosine kinase growth factor receptors). This interaction results in initiation of a series of signal transduction cascades and involves cellular phosphorylation events. Interaction of estrogen and possibly xenoestrogens with cell surface receptors results in immediate activation of several key pathways associated with cell growth, proliferation and survival. Finally, hormones can also act via non-genomic and non-receptor-mediated pathways, by interfering with steroid metabolism or through other steps of the hypothalamo-pituitary-gonadal axis (Fisher, 2004).

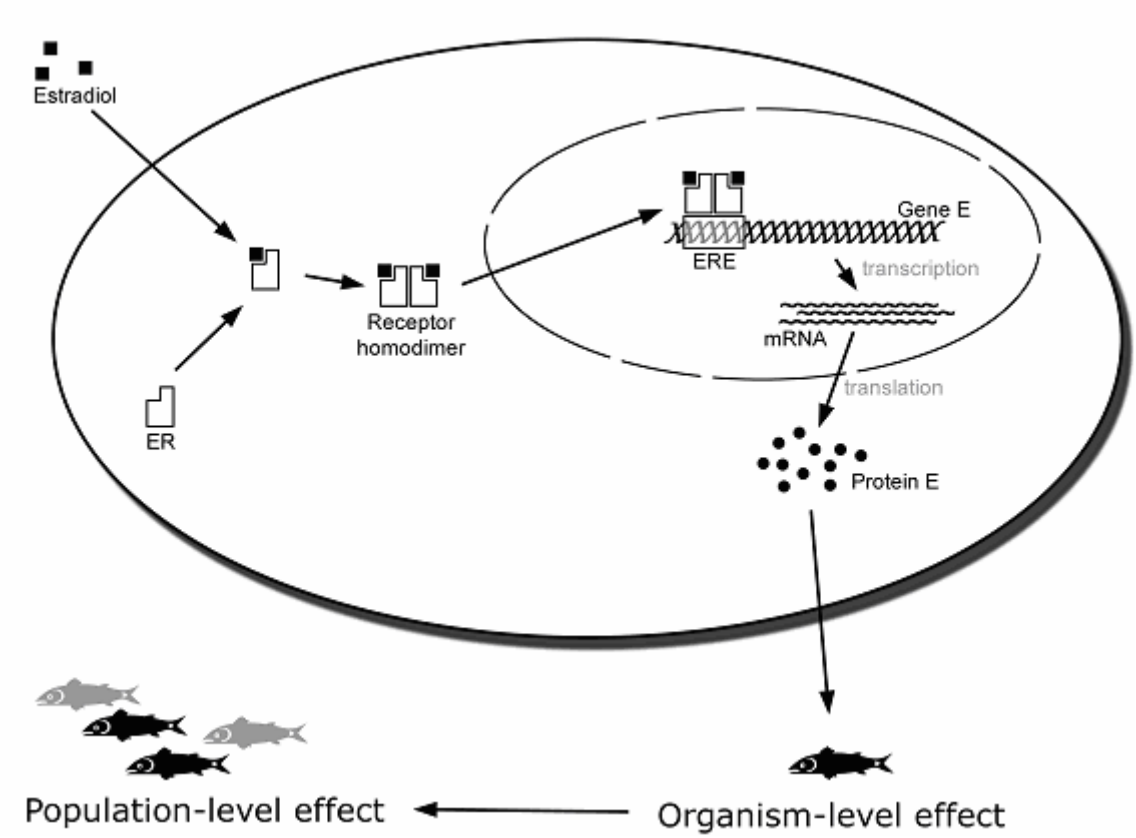

Figure 1. Schematic Representation of an Estrogen Receptor-mediated Response. ER = Estrogen receptor. ERE = Estrogen-responsive element (source: Leusch et al., 2006b).

1.1 Endocrine Disruption

There is a growing awareness of the presence of pollutants in the environment that can interfere with normal endocrine function in animals, including humans. Of particular concern are pollutants that are capable of mimicking or modulating the effects of hormones that control sexual development and reproduction (i.e. estrogens and androgens). Such chemicals interfere with normal reproductive processes and behaviour of an organism, thereby potentially affecting reproductive fitness and population dynamics (Arcand-Hoy and Benson, 1998). Numerous chemicals have been demonstrated to be endocrine-disrupting compounds (EDCs; also known as endocrine-active compounds, EACs), including natural and synthetic hormones such as estradiol, estrone and ethynylestradiol (Desbrow et al., 1998), PCBs (Andersson et al., 1999), non-ionic surfactants (Routledge and Sumpter 1996), some pesticides (e.g. atrazine, DDT), dioxins, and some metals (e.g. cadmium) (Bradley and Zacharewski, 1998; Quabius et al., 2000; Bustnes et al., 2001; Willingham, 2001; Hayes et al., 2002). The European Commission has established a priority list of 533 man-made compounds and nine synthetic and natural hormones (KIWA Water Research for GWRC, 2003).

Aquatic organisms are particularly vulnerable to the effects of EDCs as aquatic systems are a repository of chemicals derived from human activity. Effluent from sewage treatment plants (STPs) may be a significant source of EDCs to aquatic systems. Contamination of sewage

effluent with EDCs can come from natural human and animal hormones such as estradiol, testosterone and estrone, and from birth control pills containing 17a-ethynylestradiol (Purdom et al., 1994; Desbrow et al., 1998). Reported abnormalities include reduced penis size and testicular abnormalities in alligators inhabiting organochlorine-contaminated lakes (Guillette et al., 1994); reduced gonadal growth in fish near pulp mills (Munkittrick et al., 1992); and masculinization of female gastropods (e.g. the development of a penis and vas deferens) due to tributyltin, a paint additive used to reduce barnacle growth on boat hulls (Matthiessen and Gibbs, 1998). There are also numerous synthetic chemicals described above that have estrogenic properties. Studies in the UK demonstrated that effluent from STPs as well as sewage-contaminated receiving waters have estrogenic effects on fish with increased levels of vitellogenin (Vtg) in males and a high incidence of intersex, a pathological condition characterised by the presence of both male and female sex cells in the gonads (Purdom et al., 1994; Sumpter, 1995; Jobling et al., 1998). Exposure to EDCs has been linked to decreased fertility and fecundity in fish (Robinson et al. 2003; Jobling and Tyler, 2003; Ankley et al., 2005) and to reproductive abnormalities leading to infertility in humans (Gill et al., 1976; Eertmans et al., 2003)

In wastewater treatment systems the mechanisms for removal and biotransformation vary for each class of EDCs and different types of treatment processes. This depends on their physical and chemical properties and changes in treatment parameters (e.g. temperature, pH, salinity, organic matter) (Langford and Lester, 2003). Most steroid and sterol estrogens enter treatment systems in their conjugated form and are deconjugated during primary treatment (Alcock et al., 1999; Baronti et al., 2000). For example, 17b-estradiol-17-glucuronide and 17b-estradiol-3-glucuronide are cleaved to form 17b-estradiol (E2), the biologically active form. This is in turn oxidised to estrone (E1) the final transformation product in the effluent (Ternes et al., 1999). The synthetic hormone 17a-ethynylestradiol (EE2) is very resistant to the biological treatment processes in wastewater treatment plants (Ternes et al., 1999). Japanese studies showed that EE2 accounted for 34% of estrogenicity in raw sewage but for 100% in the final effluent after activated sludge treatment (Matsui et al. 2000). Studies in Europe and America have demonstrated that estrogens are being significantly, but not completely, degraded in well-operated secondary biological treatments rather than absorbed onto suspended solids (Baronti et al., 2000; Körner et al., 2000). Generally, treatment by activated sludge is fairly efficient at removing steroid hormones from the aqueous phase, with removal rates of 76-92% E2, 19-94% E1 and 83-87% EE2 of the influent concentrations (Baronti et al. 2000) due to breakdown or partitioning into solids. Despite this, endocrine-disrupting (ED) effects are still found in fish inhabiting sewage-contaminated water (Jobling et al., 1998), indicating that very small concentrations are sufficiently potent to affect aquatic biota.

Another major class of EDCs in sewage effluent is the non-ionic alkylphenol polyethoxylates (APEOs) surfactants and their primary and secondary breakdown products, alkylphenols (APs) and alkylphenol carboxylates (APECs), all of which are estrogenic (Jobling et al., 1998; Routledge et al.,1998; Matsui et al., 2000). While the oxidative cleavage of the APEOs is rapid, the resulting intermediates biodegrade more slowly because of the stability of the benzene ring and their limited water solubility. Generally, nonylphenols (NPs) are dominant components of the APs in secondary effluents and can be up to 7.6 times higher in concentration than in the primary effluent (Ahel et al., 1994). Thus their proportion of EDCs in treated effluent is significant. Johnson and Sumpter (2001) in a review of the removal of EDCs in activated

sludge treatment works concluded that despite the substantial removal of estrogens and surfactants, the remaining concentrations were still sufficient to affect exposed fish.

Environmental conditions such as temperature, pH, salinity, hydraulic retention time (HRT), and sludge retention time (SRT) have been demonstrated to affect steroid hormone removal (Langford and Lester, 2003). HRT is the period that the effluent is retained within the STP and SRT is the period that the sludge is retained in the sedimentation tank. For example, a 26-hr HRT and 20-day SRT in an STP was shown to remove >75% E2 at 13-15ºC while > 94% was removed at 18-19ºC (Johnson et al. 2000). Temperature greatly influences the removal of surfactants with greater efficiencies in the summer than in the winter. How this affects STPs in countries like Australia where temperatures are generally higher than those in Northern Hemisphere countries is unknown. This demonstrates the importance of environmental conditions on EDC-removal efficacy.

Reuse of treated sewage effluent water for various purposes is a growing trend globally (Expert Panel Review and Findings, 2002). Such water is largely used for irrigating agricultural crops, vegetable crops, and recreational fields. It is also used as potable water in some countries including parts of the United States; for example high quality reclaimed water is discharged into the Occoquan Reservoir, which supplies potable water to residents in the vicinity of Washington, D.C. Singapore also currently treats a small proportion of its sewage effluent to drinking water quality and this water is currently being discharged into reservoirs. The reservoir water is then treated for drinking purposes (i.e. indirect potable use) (NEWater) (Expert Panel Review and Findings, 2002).

1.2 Assessment of EDCs

Assessment of the risks of EDCs in the environment requires an understanding of both the fate in the environment and associated effects on the biota. While an understanding of the fate of EDCs is critical to determining the ability of treatment technologies to remove these substances, it does not give information on the effects to biota exposed to these substances. Effects-based assessment of EDC-contaminated waters is thus crucial to determining the significance of the presence of such compounds in the environment so that appropriate management strategies can be developed.

EDCs can evoke a number of effects from agonistic (estrogenic/androgenic) or antagonistic (anti-estrogenic/anti-androgenic) outcomes. Effects-based assessment requires a battery of tests ranging from *in vitro* bioassays to *in vivo* studies. *In vitro* bioassays are tests performed with individual molecules or cells. A number of *in vitro* bioassays have been established such as the yeast estrogen screen (YES), the E-Screen cell proliferation assay, and estrogen receptor (ER) competitive binding assay. These have been widely used to screen estrogenic compounds as well as EDC-contaminated waters. While *in vitro* bioassays are sensitive, specific and rapid, they have relatively less biological/ecological relevance than *in vivo* studies, which are carried out on whole organisms. *In vivo* assays such as the vitellogenin (Vtg) induction test, intersex, GSI analysis, and sexual behaviour in fish, have also been established and are sensitive to EDC effects. These too have been widely used to assess EDC effects, and a number of commercially available bioassays have been developed. *In vivo* studies will give a better indication of the effect in a population because the experimental model is a more complex

biological system (a whole organism) but are expensive and time-consuming, and results of *in vivo* experiments can sometimes be confounded by the high variability between specimens. *In vitro* bioassays on the other hand are cost- and time-effective, and because they are simplified models of the whole organism there are less variables that can confound the result of an experiment. *In vitro* methods can thus more clearly identify a subtle effect and its mechanism of action.

Ideally, a battery of complementary bioassays should be used to assess estrogenic activity of environmental samples. A combination of bioassays can provide insights into the mechanisms of action of specific endocrine disruptors (Zacharewski, 1997). However, *in vitro* bioassays can only provide limited information on the potential of a specific chemical or mixture to induce whole-organism effects, and it is therefore important to correlate *in vitro* results with *in vivo* measurements (Zacharewski, 1998). This is particularly true when dealing with endocrine disruptors that can act in unpredictable ways in whole organism systems due to the complexity of feedback mechanisms involved in endocrine communication (Hadley, 1988). Recent reviews by environmental policy agencies (Huet, 2000; NIEHS, 2002; ICCVAM 2003) suggest a range of *in vitro* and *in vivo* bioassays to determine the endocrine-disrupting potential of simple chemicals or complex environmental samples.

In August 1998, the U.S. EPA announced establishment of the Endocrine Disruptor Screening Program (EDSP) (U.S. EPA 2000), which uses a tiered approach for 1) identifying substances which have the potential to interfere with the endocrine system and 2) to confirm the potential for effects to occur and characterize these effects if present (U.S. EPA, 2000). The EDSP scope includes effects on humans and wildlife, effects on estrogen, androgen and thyroid hormone related processes, and evaluation of chemical substances. The Tier 1 screening (T1S) includes a utero-trophic screen, a Hershberger screen (male rodent-based tests for androgenic activity), a rodent pubertal female screen, a rodent pubertal male screen, estrogen and androgen receptor reporter gene screens, a fish reproduction screen and a frog metamorphosis screen. Tier 2 testing (T2T) includes a two-generation mammalian reproduction and development test and a mysid shrimp reproduction test (U.S. EPA 2000). U.S. EPA validation work is being conducted in close liaison with the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) established by the National Toxicology Program under the auspices of the National Institute of Environmental Health Sciences (NIEHS).

1.3 *In vitro* Bioassays

Several *in vitro* bioassays have been developed to measure the estrogenic activity of simple compounds or complex mixtures. Chemicals can interfere with endocrine systems in several ways, including binding to the hormone receptors, affecting the synthesis or metabolism of natural hormones, and affecting the synthesis or metabolism of the hormone receptor itself (Katzenellenbogen and Muthyala, 2003; Zacharewski, 1997). There are four main categories of *in vitro* bioassays, depending on which endpoint of the biological response to natural estrogens they measure: receptor binding assays, reporter gene assays, *in vitro* gene expression assays, and cell proliferation assays. Receptor binding assays measure estrogenicity at the molecular level, while the other three are cellular assays. The cellular assays are generally based on immortalized (transformed) cells instead of primary cells. Although immortalized cells are usually less accurate models of *in vivo* situations than primary cells, they have several advantages over

primary cells. Primary cells can survive for some time *in vitro* but are unable to grow and eventually die. Reproducibility is a major concern with primary cells, where interassay variability is greatly increased due to the variation in cell yield from the organ tissue. Primary cells are also much less convenient than immortalized cells because of the need to maintain a stock of donor organism. Finally, there is also the ethical concern of sacrificing animals for routine environmental monitoring. Immortalized cell lines are therefore more practical for *in vitro* cellular bioassay, although immortalized cell lines can sometimes lose sensitivity compared with primary cell lines (Bols et al. 2005).

Each type of bioassay has its advantages and limitations, and no single assay can provide a complete assessment of the endocrine-disrupting activity of a chemical or mixture at the organism-level. The following paragraphs provides a more detailed description of *in vitro* assays that have been developed and are in relatively common use for the evaluation of estrogenic EDCs. Appendix 1 summarizes the main characteristics of all the discussed bioassays in a table format for easier comparison.

1.4 Overview of the Project

While there are many bioassays to measure ED activity available in the literature (OECD 2001), this project will focus on the evaluation and validation of selected bioassays for estrogenicity. It is our intention to develop a battery (or "toolbox") of *in vitro* bioassays to detect and quantify estrogenic activity in environmental water matrices. This assessment is not a means to determine effects, although it could be argued that *in vitro* bioassays may provide insights into potential subsequent effects.

The challenge in assessing waters contaminated with a mixture of EDCs is that these compounds may have different modes of action on the endocrine system. It is thus imperative that we develop a toolbox of bioassays that is sufficiently comprehensive to detect estrogenic activity from a mixture of compounds and predict risk to a wide distribution of species. This will be the next step towards implementation of some of these methods for use in a regulatory framework on a global scale.

Significance of the project:

- Appropriate bioassays need to be identified to assess the complex and multiple modes of action of EDCs so that endocrine-active chemicals can be effectively managed and/or regulated. It is impractical and extremely costly to solely rely on chemical analysis of effluent due to the number of potential analytes present and because many of these substances are known to be biologically active below the analytical limit of detection. Bioassays can further provide an assessment of integrated exposure (eg. integration of synergistic and antagonistic effects), which cannot be predicted by chemical methods alone.
- The use of mechanism-based bioassays will enable rapid screening of waters as part of exposure assessment of whole waters. This may in some cases negate or justify the need for further investigation using *in vivo* methods and/or chemical identification of active substances.

It is important to note at this point that estrogenic disruption is only one type of endocrine disruption, which has received the most attention so far. However, bioassays to test for thyroid and androgenic disruption are currently under development. Although these endpoints are beyond the scope of the current project, they should eventually be included in a comprehensive bioassay battery to test for endocrine disruption.

There are four major types of in vitro assays reviewed in this document; 1) estrogen receptor binding assays, 2) reporter gene assays, 3) gene expression assays, and 4) cell proliferation assays. The following discussion provides a review of these assays. A subset of these assays will be selected for inclusion in the "toolbox". Selection of the most appropriate assays will be based on a variety of criteria (discussed in Section 6).

2.0 Estrogen Receptor Binding Assays

The most basic assay for estrogenicity is the estrogen receptor (ER) competitive binding assay. Competitive receptor binding assays measure the ability of chemicals to compete with the native hormone for binding to the receptor (ICCVAM, 2003). Binding to the receptor is the initial step of genomic steroid action (Danzo, 1997) and is a prerequisite for many subsequent cellular effects (Figure 1), such as vitellogenin (Vtg) synthesis in fish, and induction of progesterone receptors, uterine cell division and growth in mammals. There are several variations of this assay, depending on the source of the ER (synthesized as recombinant in the laboratory or isolated from animal tissue), the label attached to E2 (tritium or a fluorophore), and the receptor medium (suspended in reaction buffer or fixed to the reaction well). Estrogen receptors (ER) have been isolated from uteri or liver tissue from a variety of vertebrate species, and a large number of natural and synthetic chemicals with a range of chemical structures can bind to these receptors (Katzenellenbogen, 1995; Hong et al., 2002; Katzenellenbogen and Muthyala, 2003). To date, two main ER isoforms have been identified in mammals, ERα and ERβ (Gustafsson, 1999). A third isoform has been identified in fish, and has been labelled ERγ (Loomis and Thomas, 1999). Some isoforms are more common in some tissues than in others (Hiroi et al., 1999; Pelletier and El-Alfy, 2000; Nielsen et al., 2001), and each isoform has slightly different ligand affinities (Legler et al., 2002), which may explain some of the species- and tissue-specific variability in estrogenic responses (Matthews et al., 2000).

In some configurations, this type of bioassay can be relatively inexpensive and rapid (yielding results within a couple of hours), making it suitable for large scale monitoring and screening. However the relative binding affinity for the ER is not always a precise predictor of more complex *in vitro* and *in vivo* responses. For example, the dose required to produce a change *in vivo* may be much lower or higher than one might expect from evaluating the EC50 for ER binding due to metabolism and tissue distribution *in vivo* (Laws et al., 2000; ICCVAM, 2003; Kinnberg, 2003). Receptor binding assays also cannot discern between agonistic (excitatory) and antagonistic (inhibitory) effects (Zacharewski, 1998), and displacement ability of a mixture is a summation of absolute effects of each of its compounds. This "limitation" does however have

the benefit of avoiding false negatives when excitatory and inhibitory effects from complex mixtures might cancel each other out in more intricate cellular bioassays (Conroy et al., 2005). The following discussion provides an overview of the different types of ER binding assays currently available.

Assay No. 1: Standard ER Binding Assay (ICCVAM 2003)

In its most common incarnation, the ER binding assay is performed with tritiated E2 as the native ligand for the ER binding site. Estrogen receptors can be purchased from commercial sources such as Sigma and Invitrogen, synthesized in the laboratory from recombinant DNA (Kuiper et al., 1998; Matthews et al., 2000), or isolated from a variety of animal tissues, such as rat (*Rattus norvegicus*) uteri (ICCVAM 2003), alligator (*Alligator mississipiensis*) oviducts (Vonier et al., 1996), and livers of Atlantic salmon (*Salmo salar*) (Lazier et al*.,* 1985; Yadetie et al.,1999), carp (*Cyprinus carpio*) (Kloas et al., 2000), and frog (*Xenopus laevis*) (Lutz and Kloas, 1999).

The sample is incubated with a standard amount of receptor and radioligand in buffer. After 18-24 h of incubation, the receptors (and bound ligand) are isolated either by charcoal or hydroxyapatite (HAP) stripping, and beta radiation from the radiolabelled E2 still bound to the receptor is counted in a scintillation counter. The amount of radiolabelled E2 still bound to the receptor in the presence of increasing concentrations of the test chemical (or sample) is compared with an E2 standard curve, and the estrogenic potency of the sample is quantified in estradiol equivalents (EEq). Data interpretation is an important component for this assay. If a test chemical and the radioligand compete for a single common ER binding site, the competitive binding will present a sigmoid shaped binding curve as determined by the law of mass action (GraphPad Software Inc, Introduction to Radioligand Binding, http://www.graphpad.com/curvefit/introduction9e.htm). Specifically, the curve should descend from 90% to 10% specific binding over approximately a two-log unit change in chemical concentration. A binding curve that drops dramatically over one order of magnitude should be questioned as it likely indicates a change in the biochemical stability of the assay and not true competitive inhibition (Laws et al., 2005).

Overall, this assay is a relatively simple and direct measure of competitive displacement. It does however require the use of tritiated material in a properly designated laboratory, and generates small quantities of radioactive waste with a half-life of 12.3 years. The charcoal stripping or HAP separation step is also a source of variability, particularly with quickly dissociating ligands.

Assay No. 2: FP-ER Binding Assay (Parker et al., 2000)

In a fluorescence polarization (FP) ER binding assay, the tritiated E2 is replaced by a fluorescent analogue (fluormone). When the fluormone is bound to the receptor, it has a high polarization value. When displaced from the ER binding site, the fluormone has a low polarization value. The change in polarization is used to determine the relative affinity of the sample for the receptor binding site.

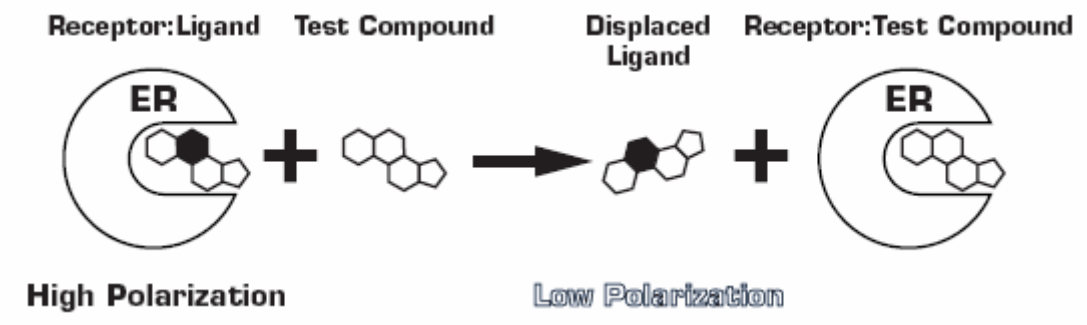

Figure 2. The Principle of Fluorescence Polarization. (source: Panvera/Invitrogen protocol L0712).

The sample is incubated with a standard amount of ER and fluormone in buffer. After 2-6 h of incubation, the polarization value is measured in a fluorescence polarization instrument, providing a ratio of bound vs free fluormone. This measure is compared with an E2 standard curve, and the estrogenic activity in the sample expressed as EEq.

This assay is similar to the standard ER binding assay discussed above, but does not create radioactive waste and avoids the need for a charcoal or HAP separation step. It is however sensitive to background fluorescence polarization from the sample, although this can be corrected by the use of appropriate blanks. Commercial kits are available from Invitrogen with human ERα (P2614) and ERβ (P2615). For this assay and others that use multi-well plates, care should be taken to assure that test chemicals are not adhering to plastic during the assay.

Assay No. 3: ELRA (Seifert, 2004)

The enzyme-linked receptor assay (ELRA) employs the same principles as competitive immunoassays based on ligand-protein interactions, with an anti-ER antibody.

Wells are pre-coated with an E2-BSA (bovine serum albumin) conjugate. The sample and a standard amount of ER is then added to the well, and incubated for 1 h (Figure 3, step 1). ER not bound to the E2-BSA coating is washed away, and a biotinylated mouse anti-ER antibody is added, and incubated for another 1 h (Figure 3, step 2). A streptavidin-POD-biotin complex is added and incubated for a further 1 h (Figure 3, step 3). Finally a luminescent substrate (luminol) is added. The substrate turnover is measured with a luminometer after 5 min of incubation.

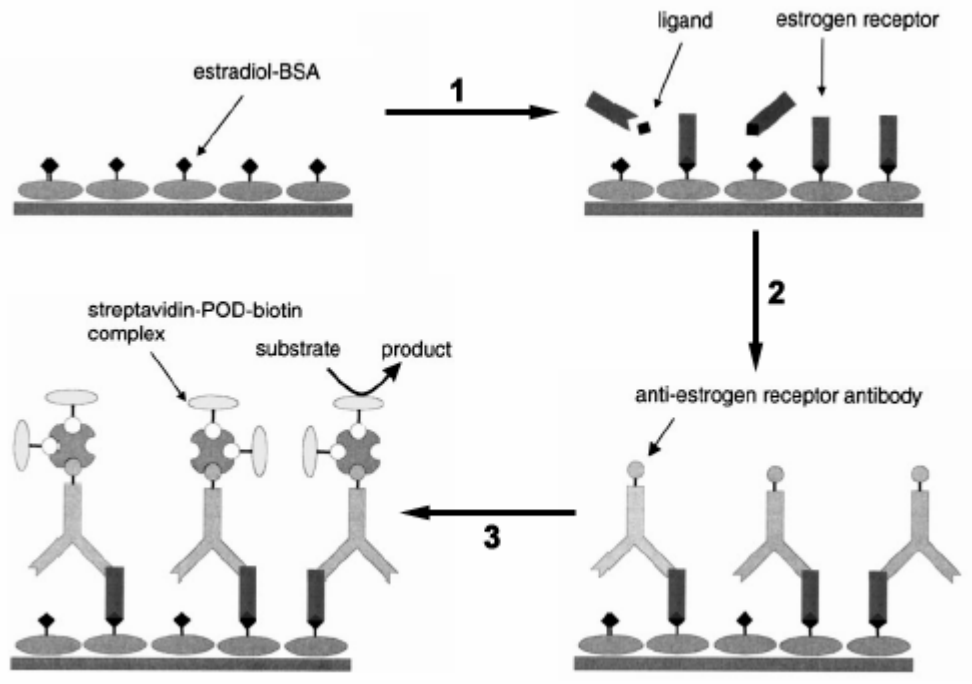

Figure 3. Principle of the ELRA. (Seifert et al., 1999)

This method initially used the chromogenic substrate tetramethylbenzidin (TMB) with an ELISA plate reader (Seifert et al., 1999), but use of a more sensitive luminescent substrate (luminol) has improved the detection limit of the assay by a factor of 5.

Receptor binding assays are simple and generally suitable to high-throughput screening, being inexpensive and relatively rapid. They are however poor predictors of whole-organism effects and cannot differentiate between agonistic and antagonistic chemicals. These assays are compared and contrasted in Appendix I, Table 1.

3.0 Reporter Gene Assays

Receptor-mediated gene induction can be measured in reporter gene assays. In this type of cellular assay, the sample is incubated with cells which produce a specific protein/enzyme upon estrogenic stimulation. Estrogenicity is thus measured by quantifying the production of the estrogen-responsive protein or enzymatic activity after incubation.

Reporter gene assays are generally conducted with genetically engineered yeast, fish, or mammalian cells transfected with an estrogen-responsive element (ERE) DNA sequence linked

to a reporter gene (Zacharewski, 1997; Kinnberg, 2003). Binding of an agonist to the receptor causes a cascade of molecular events that allows the receptor to bind to the ERE and activate the gene expression machinery (Figure 1). The product of the reporter gene can then be measured appropriately (most reporter genes are galactosidase or luciferase genes, with protein/enzymatic products easily measured by spectrophotometry and luminometry). Vertebrate-based assays are conducted with fish (Ackermann et al., 2002; Rutishauser et al., 2004) or mammalian cells (Legler et al., 1999; Vinggaard et al., 1999; Balaguer et al., 2000; Wilson et al., 2004) usually transfected with an ERE linked to the luciferase gene. Simple yeast-based assays (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998; Garcia-Reyero et al., 2001) commonly use yeast cells transfected with a plasmid containing a mammalian ERE linked to a galactosidase reporter gene. Because yeast do not posses an endogenous ER, an expression plasmid containing a mammalian ER must also be inserted. Finally, chimeric yeast reporter gene assays use chimeric receptors with a mammalian ligand binding domain and a yeast Gal4 DNA binding domain, thus harvesting the natural yeast genetic machinery (Nishikawa et al., 1999).

Reporter gene assays are susceptible to the presence of anti-estrogenic substances, which in complex mixtures can counteract the effects of agonistic chemicals and result in underestimation of the activity of the sample (Kinnberg, 2003). This can also however be an advantage, because anti-estrogenic (antagonistic) activity can be evaluated by incubating the sample with a concentration of E2 that produces a sub-maximal response. If the chemical is antagonistic, then the response will be decreased and anti-estrogenicity can thus be quantified. Reporter gene assays cannot however identify chemicals that have non-genomic receptor-mediated effects (Thomas, 2003), and thus may lead to some false negative results. The following provides an overview of the reporter gene assays currently available.

3.1 Recombinant Yeast Assays

Yeast-based reporter gene assays have been widely used to measure both the relative potency of individual compounds and the estrogenic potency of environmental samples. Yeast cells lack endogenous steroid hormone receptors, but can be stably transfected with the gene for human ER. This lack of endogenous steroid receptors allows for standardization of the ER between strains (since they all have to be transfected with the ER gene) and also avoids the issue of complex interactions between different steroid receptors (this is however also a limitation of yeast assays, precisely because they cannot account for those complex interactions). Yeast cells are more robust than mammalian cells, and thus usually less susceptible to cytotoxicity from complex mixtures. Yeast cells grow faster than mammalian cells, and they are easier to manipulate, making yeast assays more cost-effective than mammalian assays. However, yeast-based assays may lead to more false negatives than vertebrate-based cellular assays because of the basic differences between yeast and vertebrate cells where yeast contain a cell wall which may impede active and passive transport of tested chemicals to the intracellular space. The following discussion provides an overview of the yeast-based reporter gene assays.

Assay No. 4: YES (Routledge and Sumpter, 1996; De Boever et al., 2001)

In the Yeast Estrogen Screen (YES), yeast cells *Saccharomyces cerevisiae* have been stably transfected with the gene for human ERα and a plasmid containing an ERE-linked *lac-Z*

gene. Activation of the receptor by binding of an agonistic ligand causes expression of the *lac-Z* gene, which produces β-galactosidase (Figure 4).

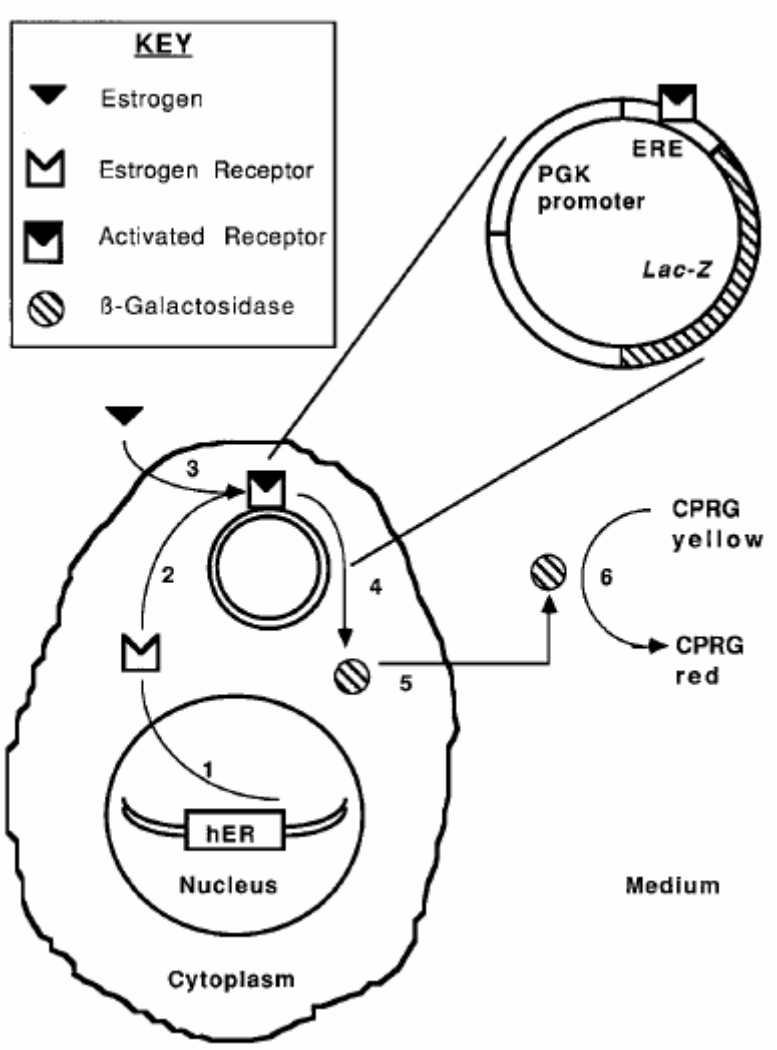

Figure 4. Principle of the YES Assay. (Routledge and Sumpter, 1996)

The yeast is distributed in a 96-well plate and exposed to the sample in culture medium for 2-3 d. The yellow chromogenic substrate CPRG (chlorophenol red-β-D-galactopyranoside) is then added and its transformation into a red product by β-galactosidase is measured in a spectrophotometer at 540 nm (note that in the original Routledge and Sumpter 1996 protocol, CPRG was added at the start of the incubation, however De Boever et al. 2001 suggest that CPRG is slightly estrogenic and recommend addition of CPRG at the end of the incubation). Galactosidase activity, a measure of the ability of the sample to induce ER-mediated gene expression, is then compared with that of a standard curve, and the estrogenic potency of the sample expressed as EEq.

The YES assay is by far the most widely used yeast-based reporter gene assay, and a lot of data for individual compounds are therefore available for this assay. It is suitably sensitive and relatively robust, but cell toxicity appears to be an issue with highly concentrated environmental aqueous samples. The assay also suffers from the usual "false negative" limitations of yeast-based assays, as discussed above.

Assay No. 5: Yeast-based Reporter Gene Assay (Gaido et al., 1997)

In this assay, yeast cells (*S. cerevisiae* strain BJ3505) are transfected with a human ERα expression plasmid and a reporter plasmid carrying two ERE upstream of the *lac-Z* gene. The transfected yeast are incubated with the sample in 5mL culture medium for 1 d. A portion of the medium is then pipetted in triplicate in a 96-well plate and the red chromogenic substrate 2-nitrophenyl-β-D-galactosidase (ONPG) is added. The formation of the orthonitrophenol, the yellow product that results from β-galactosidase cleavage of ONPG, is measured spectrophotometrically at 420 nm after a brief incubation. Galactosidase activity is compared with that of an E2 standard curve, and the estrogenicity of the sample expressed relative to the standard curve (as EEq).

Because the yeast is incubated in 50mL vials during exposure instead of a 96-well plate, this assay is more cumbersome than the YES. It also is more sensitive to cytotoxicity than the YES (Saito et al., 2002), and therefore seems less appropriate in its current form for routine monitoring of complex environmental mixtures.

Other Yeast-based Reporter Gene Assays

There are several other yeast-based reporter gene assays, but most are modifications of one of the assays described above. For example, the LYES assay is a YES assay with a lyticase enzymatic digestion step to decrease the time required by the assay (Schultis and Metzger 2004). Some assays use different ERE sequences, such as the vitellogenin ERE sequence (Graumann et al., 1999). Most yeast-based reporter gene assays use human ERα, but ER from other species can also be used, such as rainbow trout ER (Petit et al., 1997).

3.2 Chimeric Receptor Yeast-based Assays

Because yeast cells do not have endogenous ER, the coactivators that mediate signals from the receptor to the basal transcriptional machinery might differ from yeast to vertebrates. In the YES, transcriptional activation of the ER might occur by interaction of the ER with unrelated coactivation factors present in the yeast, resulting in unreliability of the test. The chimeric receptor yeast-based assays are an attempt to overcome this problem by harvesting a yeast-based receptor system (usually Gal4). Chimeric receptor yeast-based assays are therefore generally much faster than other yeast reporter gene assays, usually requiring only 4-6 h of exposure instead of several days. The following discussion provides and overview of the available chimeric receptor yeast-based assays.

Assay No. 6: Yeast Two-hybrid Assay (Nishikawa et al., 1999)

In the yeast two-hybrid assay, yeast cells (*S. cerevisiae* strain Y190) are transfected with an expression plasmid containing a hybrid transcription activator consisting of the ligand binding

domain of the ER (ERLBD) linked with a GAL4 DNA binding domain (GAL4DBD), and another plasmid containing the receptor interaction domain of coactivators (usually the TIF2 coactivator) linked with a GAL4 activation domain (GAL4AD). When a compound binds to the hybrid ERLBD/GAL4DBD, it complexes with the hybrid TIF2/GAL4AD to form a fully functional GAL4 transcription activator, which causes expression of the *lac-Z* gene and production of β-galactosidase (Figure 5).

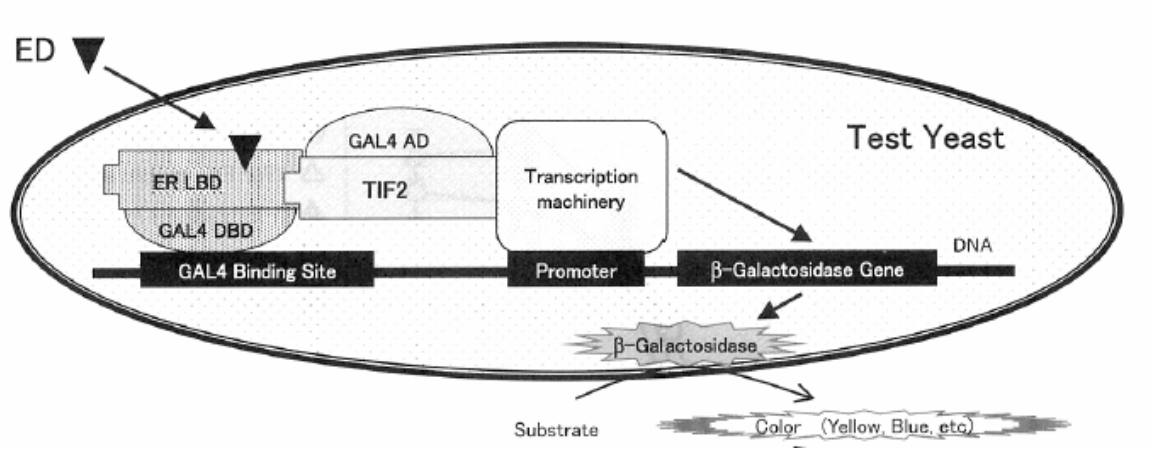

Figure 5. Principle of the Yeast Two-hybrid Assay. (Nishihara and Nishikawa, 2001)

The yeast transformants are grown overnight. A standard amount of overnight culture is then added to a 96-well plate along with fresh medium containing the sample. The yeast cells are exposed for 4 h. A β-galactosidase substrate (ONPG) is then added, and β-galactosidase activity is determined spectrophotometrically at 415 nm. The activity is compared to that of a standard curve, and expressed as EEq.

This assay is slightly less sensitive than the YES assay, and appears to be more sensitive to cytotoxicity (Nakano et al. 2002; Saito et al. 2002). It is therefore probably more appropriate for studies on model compounds and ER coactivators than for environmental monitoring.

Assay No. 7: Hybrid Receptor Yeast-based Assay (Louvion et al., 1993)

In this assay, the yeast constructs express a fusion protein carrying the hormone binding domain of the human ERα connected to the yeast GAL4 DNA binding domain. Upon binding of an appropriate ligand, the fusion protein recognizes a responsive DNA element upstream of a β-galactosidase reporter gene.

The yeast strain is grown overnight, and the culture incubated in 100mL Erlenmeyer flasks with the dissolved sample for 2h. β-Galactosidase activity is then determined by adding ONPG.

Assay No. 8: yEGFP Bioassay (Bovee et al., 2004)

The yeast Enhanced Green Fluorescent Protein (yEGFP) assay uses yeast cells that have been stably transfected with the human ERα and a yEGFP reporter gene linked to a double ERE promoter sequence incorporated directly into the yeast genome (instead of in a plasmid as is the case in the other yeast assays). This assay measures the reporter protein (GFP) directly, as opposed to other yeast-based assays which measure the reporter protein (β-galactosidase) by its activity.

Aliquots of an overnight culture are pipetted into a 96-well plate and incubated with fresh culture medium containing the sample. After 4 h of exposure, fluorescence is counted in a fluorescence plate reader using excitation at 485 nm and measuring emission at 530 nm. Fluorescence (a measure of yEGFP present in the well) is then compared with a standard curve and the estrogenicity of the sample expressed relative to that standard curve (EEq).

This assay does not require either cell disruption or the addition of a substrate, and provides results in a short time (4 h). It is thus very well adapted for high throughput screening and automation, although no studies so far report using this assay to monitor environmental water samples.

Yeast-based reporter gene assays are generally more robust than mammalian-based reporter gene assays, but less sensitive. Like receptor binding assays, yeast-based reporter gene assays are poor predictors of whole-organism effects. These assays are compared and contrasted in Appendix I, Table 1.

3.3 Recombinant Mammalian Cell Reporter Gene Assays

Reporter gene assays with mammalian cells are usually more expensive and require experienced handling and advanced laboratory equipment compared with yeast-based reporter gene assays. However, they are more sensitive than yeast-based assays and offer a more realistic representation of how mammalian cells might respond to estrogen mimics in the environment.

Assay No. 9: MVLN (Demirpence et al., 1993)

The MVLN cell line is derived from MCF-7 breast cancer cells that have been stably transfected with a plasmid containing a luciferase gene (*Luc*) downstream of an ERE derived from the *Xenopus* vitellogenin gene (MVLN stands for MCF-7-Vit-Luc-Neo, where neomycin is the antibiotic used for selection of transfected cells). MCF-7 cells have endogenous ERα and ERβ, and do not need to be transfected with an external ER. Exposure to estradiol leads to induction of the *Luc* gene. Luciferase production is then measured by addition of the substrate luciferin and quantification of luminescence in intact cells or in cell lysate with a luminometer.

The protocol described here is from the miniaturized version of the assay described in Gutendorf and Westendorf (2001). Two days prior to induction, cells are seeded into 96-well

plates. A day later, the medium is changed to steroid-free medium. On the day of induction, the medium is changed again and replaced by steroid-free medium with the sample. After 2 d of exposure, luciferin is added to the incubation medium and luciferase activity measured by luminescence plate reader. Estrogenicity is expressed relative to that of an E2 standard curve, as EEq.

Assay No. 10: MELN (Balaguer et al., 1999)

The MELN cell line is very similar to the MVLN line, but uses a stably transfected plasmid with the luciferase gene driven by an ERE in front of the β-globin promoter (MELN stands for MCF-7-ERE-βGlob-Luc-Neo).

The protocol for the MELN bioassay is similar to the MVLN bioassay, except that exposure duration is much shorter at 16-24 h, and cells are (generally) lysed before addition of luciferin. Luciferase activity is generally measured in cell lysate to achieve greater signal amplification, but the luminescent signal can also be detected from whole cells without loss of sensitivity (Fenet et al. 2003).

Assay No. 11: ER-CALUX (Legler et al., 1999)

The ER-mediated chemical-activated luciferase gene expression assay (ER-CALUX) is also very similar to the MVLN assay, except that it uses T47D breast cancer cells (instead of MCF-7 cells) stably transfected with an ERE-Luc plasmid. Like MCF-7 cells, T47D cells endogenously express both ERα and ERβ. The protocol for this assay is very similar to the MELN assay. Cells are lysed at the end of the exposure period (24 h), and luciferase activity measured after addition of luciferin.

This assay has been quite extensively used by research groups in the Netherlands, and appears to be quite robust and appropriate for environmental monitoring. It can however be slightly more expensive than the other bioassays, as a fee has to be paid to the patent holder for every use.

Assay No. 12: T47D-KBluc (Wilson et al., 2004)

This relatively new assay uses T47D cells stably transfected with a triplet ERE-promoter-luciferase reporter gene construct (available as CRL-2865 from American Type Culture Collection – ATCC).

The protocol for the assay is very similar to the ER-CALUX. Cells are seeded in 96-well plates for 24 h, after which cells are exposed to the samples for a further 24 h. At the end of the incubation period, cells are lysed and luciferase activity measured by luminescence. Although more recently established, the assay appears slightly more sensitive than the ER-CALUX and the cell line is freely available.

Assay No. 13: BG1Luc4E2 (Rogers and Denison, 2000)

In this assay (also known as the E-CALUX, Kojima et al., 2005), human ovarian cancer cells (BG-1) are used instead of breast cancer cells. The cells have been stably transfected with a

plasmid containing a single ERE upstream of a promoter (in this case the mouse mammary tumour viral promoter, MMTV) linked to the luciferase gene. The assay principle is the same as for the previous assays. Cells are cultured in 24-well plates for 7 d. At 90% confluence, the cells are exposed to the sample for 24 h. After exposure, the cells are lysed and luciferase activity after addition of luciferin is measured in a luminometer.

Although described for a 24-well plate, this assay is likely to be adaptable to a 96-well plate without loss of sensitivity, as was the case with both the MVLN and HGELN assays (Gutendorf and Westendorf 2001), although this remains untested.

Assay No. 14: HELNα and HELNβ (Balaguer et al., 1999)

MCF-7 cells possess a mix of endogenous ERα and ERβ. Chemicals exhibit subtly different affinities for different subtypes of ER (Gutendorf and Westendorf, 2001). Human uterine cervix cancer (HeLa) cells do not endogenously express ER, and can be transfected with plasmids encoding ERα or ERβ, and can therefore be used to test ERα and ERβ-mediated gene expression independently. In this assay, HeLa cells are stably transfected with a luciferase gene reporter plasmid (an ERE-βGlob-Luc-Neo plasmid, the same plasmid as in MELN cells) and a plasmid for human ERα (in the case of HELNα) or human ERβ (in the case of HELNβ). The assay protocol for this assay is the same as in the MELN assay, described above.

This assay is particularly useful to investigate differential effects of estrogenic chemicals on different isoforms of the ER. It is perhaps more adapted to comparative endocrinology research than to environmental monitoring.

Assay No. 15: HGELN (Balaguer et al., 1999)

The HGELN cell line is a HeLa cell line that has been stably transfected with a Gal4-HEGO expression plasmid (which codes for a chimeric receptor consisting of a human ER ligand binding domain and a Gal4 DNA binding domain) and a p17m5-βGlob-Luc plasmid (a repeat of five consecutive Gal4 response elements linked to a β-globin promoter sequence upstream of the luciferase gene). HGELN stands for HeLa cells with Gal4-ER-Luc-Neo. Estradiol binds to the ER ligand binding domain of the chimeric receptor, transforming the receptor into a high affinity Gal4 DNA binding homodimer. The activated complex is directed to the reporter gene construct where it binds to the Gal4 response element (17m), thus inducing luciferase gene expression. Because no mammalian proteins are known to bind and initiate gene expression via the Gal4 response element, production of luciferase is initiated exclusively by the chimeric Gal4-HEGO receptor, further increasing specificity of the response.

The protocol described here is from the miniaturized version of the assay described in Gutendorf and Westendorf (2001). Two days prior to induction, HGELN cells are seeded into 96-well plates. A day later, the medium is changed to steroid-free medium. On the day of induction, the medium is changed again and replaced by steroid-free medium with the sample. After 48 h of exposure, luciferase activity is measured by addition of luciferin to the incubation medium.

Assay No. 16: MCF7-ERE-GFP (Miller et al., 2000)

While most other mammalian reporter gene assays use luciferase as reporter, the MCF7-ERE-GFP assay uses a green fluorescent protein (GFP) reporter protein. In this assay, MCF-7 breast cancer cells are stably transfected with a GFP reporter plasmid consisting of two sequential ERE coupled to a promoter (in this case human phosphoglycerate kinase, PGK) upstream of the GFP gene. In its current form however, the MCF7-ERE-GFP assay is very time intensive, and may not be appropriate for routine monitoring.

Transiently Transfected Cells

Although transiently transfected cells can be more sensitive than stably transfected cells, there is generally more variability between assays with transiently transfected cells (due to variation in transfection efficiency). Stably transfected cell lines have therefore become more common in monitoring application, while transiently transfected cells are used in research applications because of their versatility. Some laboratories have however used transient transfected cells to determine estrogenicity of environmental samples, and compensated for variations in transfection efficiency by adding an internal control plasmid (such as a β-galactosidase expression plasmid).

Assay No. 17: E2 Bioassay (Zacharewski, et al., 1995)

The E2 Bioassay is an example of a transiently transfected cell bioassay. The assay is based on a chimeric receptor protein concept similar to that used for HGELN cells. In the E2 Bioassay, MCF-7 breast cancer cells are transiently transfected with a β-galactosidase expression vector, a p17m5-βGlob-Luc plasmid (a repeat of five Gal4 response elements linked to a β-globin promoter sequence upstream of the luciferase gene, as in the HGELN cells), and a Gal4-HEGO expression plasmid (coding for a chimeric receptor with the human ER ligand binding domain and a Gal4 DNA binding domain). Using a chimeric receptor with a Gal4 response element absent in mammalian cells avoids overlap with the endogenous ER present in MCF-7 cells, and ensures that luciferase induction is initiated exclusively by the chimeric receptor. β-Galactosidase activity is also measured and used as an internal control to normalize luminescence for transfection efficiency.

MCF-7 cells are transfected with all three plasmids. A day after transfection, the plates are washed and the buffer replaced with medium containing the sample. The cells are harvested after 24 h of exposure, and luciferase activity is measured after addition of luciferin.

Other Transiently Transfected Cell Assays

Other transiently transfected cell assays have generally been used to study differences between receptors from various species or activity of model compounds. Cells can be transfected using commercially available transfection agents (such as lipofectamine), electroporation, or by calcium phosphate precipitation. A variety of cells have been used in transient transfection, such as MCF-7 human breast cancer cells (Matthews et al., 2002), HeLa human uterine cervix cancer (Gong et al., 2003), HepG2 human hepatoma cells and U2 human osteogenic sarcoma cells (Yoon et al., 2000). The great advantage of transient transfected cells is that they are very

customisable. Instead of luciferase, other reporter genes can be engineered, such as chloramphenicol acetyltransferase (CAT) (Tully et al., 2000). Others have transfected MCF-7 cells with chimeric receptors made up of the ER ligand binding domain from several species (human, mouse, chicken, green anole, *Xenopus*, and rainbow trout) and the Gal4 DNA binding domain to compare the ability of model compounds to induce ER-mediated gene expression in different species (Matthews et al., 2002). Some assays attempt to simulate metabolic biotransformation in whole organisms by including a metabolic activation step with S9 liver microsomes (Sumida et al., 2001). Overall, transient transfections are an invaluable tool for research, but the associated inherent variability makes intra- and interlaboratory comparisons unreliable. Stably transfected cell systems may thus be more appropriate in an environmental monitoring framework.

Mammalian-based reporter gene assays generally require a high level of expertise and specialized laboratory facilities, and can therefore be relatively expensive to run. They are however rapid, more sensitive than yeast-based assays, and are better predictors of mammalian effects. These assays are compared and contrasted in Appendix I, Table 1.

3.4 Recombinant Fish Cell Reporter Gene Assay

Immortalized fish cell lines appear not to express endogenous ER (Chen et al., 2004). Engineered reporter gene systems using immortalized fish cell lines must therefore also transfect an ER expression plasmid to restore estrogen responsiveness.

Assay No. 18: RTG-2 Cells (Fent 2001)

In this transient transfection assay, a cell line isolated from juvenile rainbow trout gonad cells (RTG-2) is co-transfected with a rainbow trout ER expression vector, an estrogen-inducible reporter plasmid (consisting of a firefly luciferase gene upregulated by an ERE), and a control vector for normalising the transfection efficiency (*Renilla* luciferase).

RTG-2 cells are transfected with all three plasmids for 3 h, and then incubated for 3 d with the samples in culture medium. After 3 d of exposure, the cells are lysed, and firefly luciferase activity is measured. Firefly luciferase is then quenched, and *Renilla* luciferase activity is measured. Firefly luciferase activity (a measure of estrogenicity) is then corrected by *Renilla* luciferase activity (a measure of transfection efficiency), and estrogenicity quantified by comparison with luciferase activity of an estradiol standard curve.

The RTG-2 bioassay is a better predictor of potential piscine effects compared with other assays. It is however inherently more variable and requires a high level of technical expertise. This assay is compared and contrasted with other bioassays presented in this review in Appendix I, Table 1.

4.0 Gene Expression Assays

Some cells (such as fish primary hepatocytes or breast cancer cells) are naturally estrogen-responsive. In other words, these cells naturally express a protein(s) under estrogenic stimulation, such as vitellogenin in fish hepatocytes (Petit et al., 1997; Tremblay and Van Der Kraak, 1998), alkaline phosphatase (ALP) in Ishikawa cells (Matsuoka et al., 2005), or pS2 protein in breast cancer cells (Rosenberg Zand et al., 1999). If the estrogen-mediated protein can be measured, this endogenous gene expression can be used *in vitro* to detect estrogenic activity in a sample. Induction of the estrogen-responsive proteins can also occur via non-ER mediated pathways (Zacharewski, 1997), which may lead to false positives (depending on the exact definition of estrogenicity). The results of these cell-specific assays may also not be relevant to other tissues or species. Nevertheless, they do provide a measure of a natural cellular response to estrogenic stimulation.

Assay No. 19: Ishikawa cell-ALP (Matsuoka et al., 2005)

The Ishikawa cell line, a human endometrial cancer cell line, produces alkaline phosphatase (ALP) when exposed to estrogens.

The cells are seeded in a 24-well plate and incubated for 24 h. The cells are then exposed to the samples in culture medium for 3 d. The cells are then lysed, *p*-nitrophenyl phosphate is added, and ALP activity is measured by monitoring formation of *p*-nitrophenol spectrophotometrically at 405 nm. ALP activity in the sample is then compared with an estradiol standard curve, and estrogenicity expressed in estradiol equivalents (EEq).

This assay has also been miniaturized to 96-well plate (De Naeyer et al., 2005). It appears that sensitivity of the assay decreases after 25 passages (Matsuoka et al., 2005), and a careful cell maintenance approach is required for reliable determination of estrogenicity.

Assay No. 20: BT-474 pS2 Assay (Rosenberg Zand et al., 1999)

The pS2 protein pS2 is an estrogen-regulated protein expressed in breast cancer cells (as well as normal breast epithelium). Exposure of BT-474 breast cancer cells to estradiol results in a significant production of pS2 protein, which can be measured by immunoassay.

Cells are seeded in a 24-well plate. After 24 h, they are exposed to the samples in culture medium for 7 d. After exposure, the supernatant is harvested and pS2 concentration is measured using an immunofluorometric technique (Rosenberg Zand et al., 1999) or a commercially available immunoradiometric assay. Other breast cancer cells, such as MCF-7, may also be used in this assay (Kim et al., 2004).

Assays No. 21 and 22: Primary Hepatocyte Assay

Vitellogenin (Vtg) is produced in the liver of oviparous vertebrates under estrogenic stimulation (Denslow et al., 1999), and *in vitro* production of Vtg in primary cultures of isolated

liver cells (hepatocytes, explanted directly from a donor organism) has been used as measure of estrogenicity (Tollefsen et al., 2003).

In the HEP-Vtg assay (**Assay No. 21**), hepatocytes are isolated and plated as a monolayer culture into 24-well plates for 24 h. The medium is then replaced with medium containing the sample, and the hepatocytes incubated for 4 d (with renewal of the culture medium/sample after 2 d). The medium is then analysed for Vtg content using a specific enzyme linked immunosorbent assay (ELISA). This assay uses primary cultures of hepatocytes (HEP) isolated from Atlantic salmon livers, although different species have also been used, including rainbow trout (Tremblay and Van Der Kraak, 1998), brown trout (Pessala et al., 2004), Japanese medaka (Kordes et al., 2002), catfish (Toomey et al., 1999), tilapia (Kim and Takemura, 2003), and sturgeon (Latonnelle et al., 2002).

Although Vtg induction is considered by many to be the gold standard of estrogenicity in water, other estrogen-mediated proteins such as zona radiata (Zrp) can also be monitored as indicators of exposure to estrogenic chemicals (Rutishauser et al., 2004). *In vivo*, Zrp induction appears slightly more sensitive than Vtg induction (Arukwe et al., 1997). Both zonagenesis (zona radiata protein synthesis) and vitellogenesis (yolk protein synthesis) are integral aspects of fish oogenesis (Arukwe et al., 2000). In HEP-Zrp assay (**Assay No. 22**), hepatocytes are isolated from immature rainbow trout, seeded into 24-well plates, and cultured for 24 h. Fresh medium with the sample is then added to the well and incubated for 72 h. The medium is then collected and Zrp is measured by ELISA.

The two assays described above are based on primary cells, which are more variable than immortalized cells. There are several immortalized fish liver cell lines, but sadly they appear to lose the ability to produce Vtg upon exposure to estrogenic stimulation (Bols et al. 2005). Gagné and Blaise (2000) report some success in inducing Vtg production with RTH-149 rainbow trout hepatoma cells, but at relatively high estradiol concentrations (>0.1 μM), levels that are not relevant to environmental conditions. It has been suggested that this lack of estradiol sensitivity is due to a failure of immortalized fish cells to express endogenous ER (Chen et al., 2004). If that were the case, this could be corrected by genetically engineering a stably transfected RTH-149 clone with an ER expression plasmid, thereby restoring high expression of cellular ER. To our knowledge however, such a system does not currently exist.

Gene expression assays can detect receptor-, non-receptor-, and non-genomic effects. However, they usually require longer exposure times and the response in the assay may not always be specific to estrogenic stimulation, leading to a potential for false positives. The results are also often cell-specific, and may also not be extrapolated to other tissues or species. These assays are compared and contrasted in Appendix I, Table 1.

5.0 Cellular Proliferation Assays

Cell proliferation assays are based on cell lines that require estrogen (and other factors) for growth. Such cell lines may be cancerous cell lines (such as breast cancer cells) or may be genetically engineered to be estrogen dependent (such as the yeast PL3 bioassay).

Assay No. 23: E-Screen (Körner et al., 1999; Soto et al., 1995)

The E-Screen uses human breast cancer cells, which are estrogen-dependent for growth. In this assay, the number of cells present after 5 d of exposure to a sample is compared with the number of cells present in an estradiol standard curve.

Breast cancer cells are seeded in 24- (Soto et al., 1995) or 96-well plates (Körner et al., 1999) in steroid-free medium. After 24 h, the medium is exchanged for fresh steroid-free medium with the sample. After 5 d of exposure, the number of cells in each well is determined either by counting the cells directly in a Coulter counter or with a commercially available kit (such as Promega's CellTiter 96 AQueous One Solution), or indirectly by measuring total protein content (Skehan et al., 1990). Generally the MCF-7 cell line is used, although the T47-D cell line has been shown to be equally sensitive (Matsuoka et al., 2005).

This assay was further optimised by Rasmussen and Nielsen (2002). Although this assay provides a measure of estrogenic activity at the cellular levels incorporating both genomic and non-genomic effects, there is considerable variation between different MCF-7 cell lines, with the MCF-7 BUS stock showing the highest proliferative effect under estradiol stimulation (Villalobos et al., 1995). The E-Screen assay could lead to false positives, as cell growth can be induced by a range of mitogens, cytokines, growth factors, nutrients and hormones other than estrogens (Kinnberg 2003). The E-Screen assay is more expensive and time consuming than other assays, limiting its application for large-scale screening (Kinnberg, 2003).

Assay No. 24: Yeast PL3 Growth Test (Connor et al., 1996)

The URA3 gene encodes for orotidine-5'-phosphate decarboxylase (OMPdecase), an enzyme involved in uracil synthesis. Yeasts that are deficient in this enzyme fail to grow on minimal medium, unless that medium is supplemented with uracil. The recombinant yeast strain PL3 has been stably transfected with a human ER expression plasmid and a URA3 plasmid regulated by three tandem ERE. When exposed to estrogenic chemicals, the recombinant yeast will produce OMPdecase, and thus be able to grow on uracil-deficient medium.

Yeast cells are grown on leucine, histidine, and uracil supplemented minimal medium. Once well-developed, individual colonies are resuspended in water and spotted onto minimal (uracil deficient) medium plates supplemented with the sample, and incubated at 30°C. Photos of the plates are then taken every 24 h (usually up to 5 d), and growth is compared with that of an estradiol standard curve. Alternatively, OMPdecase activity can be measured (instead of growth) after 2 d (Zacharewski, 1997). This assay is however labor and time intensive, and requires some operator familiarity or specialized expertise. This may increase its inherent inter-laboratory variability.

Cell proliferation assays (in particular the E-Screen) measures estrogenicity at the cellular level, incorporating both genomic and non-genomic effects. There are however significant issues with reproducibility that have to be addressed before they can be used in a regulatory framework. These assays can be time-consuming, limiting their application to high-throughput screening. These assays are compared and contrasted in Appendix I, Table 1.

6.0 Summary and Discussion

Different bioassays provide a measure of estrogenicity at different levels of biological organization:

- *Receptor binding assays measure estrogenic activity at the molecular level, are rapid and amenable to high-throughput screening, but are poor predictors of whole-organism effects.*
- *Yeast-based reporter gene assays are robust and rapid, but are less sensitive than mammalian-based assays, and are poor predictors of whole-organism effects.*
- *Mammalian-based reporter gene assays are rapid and very sensitive at measuring estrogenic activity, but require a high level of technical expertise.*
- *Gene induction assays can measure several estrogenic pathways at once, but require longer exposure times and may not be specific to estrogenic activity only, possibly leading to false positives.*
- *Cell proliferation assays also measure several estrogenic pathways at once, but likewise require longer exposure times and are not always specific to estrogenic activity, possibly leading to false positives.*

A comprehensive toolbox of bioassays is thus required to detect and measure estrogenic activity via different pathways.

In *in vitro* tests, parts (cells or organs) of whole organisms are used. While this approach allows researchers to detect and measure estrogenic activity without the ethical cost of maintained animal experimentation, organisms are more complicated than just the sum of their individual parts. Complex hormonal (endocrine) communication between different cells and endocrine glands within the body mean that effects on the endocrine system cannot be wholly explained through *in vitro* testing. *In vivo* assays are more expensive and time-consuming than *in vitro* bioassays, but incorporate important biological processes that are limited *in vitro*, such as metabolic biotransformation, active transport mechanisms, and complex hormonal feedback systems involved in endocrine homeostasis. Furthermore, while *in vitro* bioassays are mostly

focused to ER-mediated effects, *in vivo* assays also evaluate estrogenic activity via non ER-mediated pathways (such as interference with steroid and receptor biosynthesis) and provide information on the particular susceptibilities of the different organisms to estrogenic modulation. Several *in vivo* bioassays are presented in Appendix II.

There are several factors that need to be considered during the development of a comprehensive *in vitro* screening battery. First, no single bioassay can provide a complete assessment of the estrogenic potential of a sample; a weight-of-evidence approach must be used. Understanding the limitations and advantages of each assay is key to correct data interpretation. Second, the candidate bioassays must limit the occurrence of false negatives. False positives may be acceptable, as these would be removed in subsequent *in vivo* tests. And finally, the performance of the assay must not be dependent on laboratory-specific variables.

Selection of the bioassay to include in the toolbox is currently underway. The selection process, which will involve inter-laboratory testing and validation, will test reliability (accuracy), robustness, reproducibility (between-run precision), and repeatability (within-run precision) of each assay in different environmental water matrices (raw and treated sewage, river water, groundwater, and source water). At the end of the inter-laboratory testing, a recommendation for a comprehensive toolbox of in vitro bioassays will be made.

The toolbox would be appropriate to screen various environmental water matrices for potential estrogenic activity and help prioritise further research and monitoring efforts. In vitro bioassays cannot accurately predict whole organism effects, and the toolbox will not be an alternative to in vivo bioassays.

7.0 References

Ackermann GE, Brombacher E, and Fent K. 2002. Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents. Environmental Toxicology and Chemistry 21: 1864-1875.

Ahel M, Giger W, and Kock M. 1994. Behaviour of alkylphenol ethoxylate surfactants in the aquatic environment. I. Occurrence in transformation in sewage treatment. Water Research 28: 1131-1142.

Alcock RE, Sweetman A, and Jones KC. 1999. Assessment of organic contaminant fate in waste water treatment plants I: Selected compounds and physicochemical properties. Chemosphere 38: 2247-2262.

Andersson PL, Blom A, Johannisson A, Pesonen M, Tysklind M, Berg AH, Olsson PE, and Norrgren L. 1999. Assessment of PCBs and hydroxylated PCBs as potential xenoestrogens: In vitro studies based on MCF-7 cell proliferation and induction of vitellogenin in primary culture of rainbow trout hepatocytes. Archives of Environmental Contamination and Toxicology 37: 145-150.

Ankley GT, Jensen KM, Durhan EJ, Makynen EA, Butterworth BC, Kahl MD, Villeneuve DL, Linnum A, Gray LE, Cardon M, and Wilson VS. 2005. Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (Pimephales promelas). Toxicological Sciences 86: 300-308.

Arcand-Hoy LD, and Benson WH. 1998. Fish reproduction: An ecologically relevant indicator of endocrine disruption. Environmental Toxicology and Chemistry 17: 49-57.

Arukwe A, Celius T, Walther BT, and Goksøyr A. 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (Salmo salar). Aquatic Toxicology 49(3): 159-170.

Arukwe A, Knudsen FR, and Goksøyr A. 1997. Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. Environmental Health Perspectives 105(4): 418-422.

Balaguer P, Fenet H, Georget V, Comunale F, Terouanne B, Gilbin R, Gomez E, Boussioux AM, Sultan C, Pons M, Nicolas JC, and Casellas C. 2000. Reporter cell lines to monitor steroid and antisteroid potential of environmental samples. Ecotoxicology 9: 105-114.

Balaguer P, François F, Comunale F, Fenet H, Boussioux AM, Pons M, Nicolas JC, and Casellas C. 1999. Reporter cell lines to study the estrogenic effects of xenoestrogens. Science of the Total Environment 233: 47-56.

Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, and Samperi R. 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environmental Science and Technology 34: 5059-5066.

Bols NC, Dayeh VR, Lee LEJ, and Schirmer K. 2005. Chapter 2. Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. In Biochemistry and Molecular Biology of Fishes (T. P. Mommsen and T. W. Moon, eds.), Vol. 6. Elsevier, Amsterdam, The Netherlands.

Bovee TFH, Helsdingen RJR, Koks PD, Kuiper HA, Hoogenboom RLAP, and Keijer J. 2004. Development of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein. Gene 325: 187-200.

Bradley EG, and Zacharewski TR. 1998. Exoestrogens: Mechanisms of action and strategies for identification and assessment. Environmental Toxicology and Contamination. 17: 3-14.

Brooks BW, Foran CM, Peterson BN, Weston J, La Point TW, and Huggett DB. 2003. Linkages between population demographics and municipal effluent estrogenicity. Bulletin of Environmental Contamination and Toxicology 71: 504-511.

Bustnes JO, Bakken V, Erikstad KE, Mehlum F, and Skaare JU. 2001. Patterns of incubation and nest-site attentiveness in relation to organochlorine (PCB) contamination in glaucous gulls. Journal of Applied Ecology 38: 791-801.

Cargouet M, Perdiz D, Mouatassim-Souali A, Tamisier-Karolak S, and Levi Y. 2004. Assessment of river contamination by estrogenic compounds in Paris area (France). Science of the Total Environment 324: 55-66.

Chen MJ, Chiou PP, B.Y. Y, Lo HC, Son JK, Hendricks J, Bailey G, and Chen TT. 2004. Development of rainbow trout hepatoma cell lines: Effect of pro-IGF-I EA4-peptide on morphological changes and anchorage-independent growth. In Vitro Cellular & Developmental Biology - Animal 40: 118-128.

Connor K, Howell J, Chen I, Liu H, Berhane K, Sciaretta C, Safe S, and Zacharewski T. 1996. Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses in vivo and in vitro. Fundamental and Applied Toxicology 30: 93-101.

Conroy O, Quanrud DM, Ela WP, Wicke D, Lansey KE, and Arnold RG. 2005. Fate of wastewater effluent hER-agonists and hER-antagonists during soil aquifer treatment. Environmental Science & Technology 39: 2287-2293.

Coors A, Jones PD, Glesy JP, and Ratte HT. 2004. Assessing the elimination of estrogenic activity in advanced wastewater treatment with a reporter gene-based bioassay. Water Science and Technology 50: 181-188.

Danzo BJ. 1997. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding and physiological ligands to steroid receptors and binding proteins. Environmental Health Perspectives 105: 294-301.

De Boever P, Demare W, Vanderperren E, Cooreman K, Bossier P, and Verstraete W. 2001. Optimization of a yeast estrogen screen and its applicability to study the release of estrogenic isoflavones from a soygerm powder. Environmental Health Perspectives 109(7): 691-697.

De Naeyer A, Pocock V, Milligan S, and De Keukeleire D. 2005. Estrogenic activity of a polyphenolic extract of the leaves of Epimedium brevicornum. Fitoterapia 76: 35-40.

Demirpence E, Duchesne MJ, Badia E, Gagne D, and Pons M. 1993. MVLN cells: a bioluminescent MCE-7-derived cell line to study the modulation of estrogenic activity. Journal of Steroid Biochemistry and Molecular Biology 46: 355-364.

Denslow ND, Chow MJ, Kroll KJ, and Green L. 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8: 385-398.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, and Waldock M. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. Environmental Science and Technology 32: 1549-1558.

Drewes JE, Hemming J, Ladenburger SJ, Schauer J, and Sonzogni W. 2005. An assessment of endocrine disrupting activity changes during wastewater treatment through the use of bioassays and chemical measurements. Water Environment Research 77: 12-23.

Eertmans F, Dhooge W, Stuyvaert S, and Comhaire F. 2003. Endocrine disruptors: effects on male fertility and screening tools for their assessment. Toxicology In Vitro 17: 515-524.

Expert Panel Review and Findings 2002. Singapore water reclamation study. 24pp. (available online at http://www.pub.gov.sg/NEWater_files/download/review.PDF).

Fenet H, Gomez E, Pillon A, Rosain D, Nicolas JC, Casellas C, and Balaguer P. 2003. Estrogenic activity in water and sediments of a French river: Contribution of alkylphenols. Archives of Environmental Contamination and Toxicology 44: 1-6.

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

Fisher JS. 2004. Are all EDC effects mediated via steroid hormone receptors? Toxicology 205: 33-41.

Furuichi T, Kannan K, Giesy JP, and Masunaga S. 2004. Contribution of known endocrine disrupting substances to the estrogenic activity in Tama River water samples from Japan using instrumental analysis and in vitro reporter gene assay. Water Research 38: 4491-4501.

Fukazawa H, Watanabe M, Shiraishi F, Shiraishi H, Shiozawa T, Matsushita H, and Terao Y. 2002. Formation of chlorinated derivatives of bisphenol A in waste paper recycling plants and their estrogenic activities. Journal of Health Science 48: 242-249.

Gagné F, and Blaise C. 2000. Evaluation of environmental estrogens with a fish cell line. Bulletin of Environmental Contamination and Toxicology 65: 494-500.

Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, and McDonnell DP. 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicology and Applied Pharmacology 143: 205-212.

Garcia-Reyero N, Grau E, Castillo M, De Alda MJL, Barceló D, and Piña B. 2001. Monitoring of endocrine disruptors in surface waters by the yeast recombinant assay. Environmental Toxicology and Chemistry 20: 1152-1158.

Gill WB, Schumacher GF, and Bibbo M. 1976. Structural and functional abnormalities in the sex organs of male offspring of mothers treated with diethylstilbestrol (DES). Journal of Reproductive Medicine 16: 147-153.

Gong YH, Chin HS, Lim LSE, Loy CJ, Obbard JP, and Yong EL. 2003. Clustering of sex hormone disruptors in Singapore's marine environment. Environmental Health Perspectives 111: 1448-1453.

Graumann K, Breithofer A, and Jungbauer A. 1999. Monitoring of estrogen mimics by a recombinant yeast assay: synergy between natural and synthetic compounds? Science of the Total Environment 225: 69-79.

Guillette LJ, Gross TS, Masson GR, Matter JM, Percival HF, and Woodward AR. 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environmental Health Perspectives 102: 680-688.

Gustafsson JA. 1999. Estrogen receptor beta - a new dimension in estrogen mechanism of action. Journal of Endocrinology 163: 379-383.

Gutendorf B, and Westendorf J. 2001. Comparison of an array of *in vitro* assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166: 79-89.

Hadley ME. 1988. *Endocrinology*. Prentice Hall, Englewood Cliffs, NJ, USA.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, and Vonk A. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences 99: 5476-5480.

Hiroi H, Inoue S, Watanabe T, Goto W, Orimo A, Momoeda M, Tsutsumi O, Taketani Y, and Muramatsu M. 1999. Differential immunolocalization of estrogen receptor alpha and beta in rat ovary and uterus. Journal of Molecular Endocrinology 22(1): 37-44.

Hong HX, Tong WD, Fang H, Shi LM, Xie Q, Wu J, Perkins R, Walker JD, Branham W, and Sheehan DM. 2002. Prediction of estrogen receptor binding for 58,000 chemicals using an integrated system of a tree-based model with structural alerts. Environmental Health Perspectives 110: 29-36.

Huet MC. 2000. OECD activity on endocrine disrupters test guidelines development. Ecotoxicology 9: 77-84.

ICCVAM. 2003. ICCVAM evaluation of i*n vitro* test methods for detecting potential endocrine disruptors: estrogen receptor and androgen receptor binding and transcriptional activation assays, p. 320 pp. National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA.

Itoh S, Ueda H, Naasaka T, Nakanishi G, and Sumitomo H. 2000. Evaluating variation of estrogenic effect by drinking water chlorination with the MVLN assay. Water Science and Technology 42: 61-69.

Jobling S, Nolan M, Tyler C, Brighty G, and Sumpter J. 1998. Widespread sexual disruption in wild fish. Environmental Science and Technology 32: 2498-2506.

Jobling S, and Tyler CR. 2003. Endocrine disruption in wild freshwater fish. Pure and Applied Chemistry 75: 2219-2234.

Johnson A, Belfroid A, and Di Corcia A. 2000. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Science of the Total Environment 256: 163-173.

Johnson A, and Sumpter J. 2001. Removal of endocrine-disrupting chemicals in activated sludge treatment works. Environmental Science and Technology 35: 4697-4703.

Katzenellenbogen J. 1995. The structural pervasiveness of estrogenic activity. Environmental Health Perspectives 103: 99-101.

Katzenellenbogen J, and Muthyala R. 2003. Interactions of exogenous endocrine active substances with nuclear receptors. Pure and Applied Chemistry 75: 1797-1817.

Kim BH, and Takemura A. 2003. Culture conditions affect induction of vitellogenin synthesis by estradiol-17 beta in primary cultures of tilapia hepatocytes. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 135: 231-239.

Kim IY, Shin JH, Kim HS, Lee SJ, Kang IH, Kim TS, Moon HJ, Choi KS, Moon A, and Han SY. 2004. Assessing estrogenic activity of pyrethroid insecticides using in vitro combination assays. Journal of Reproduction and Development 50: 245-255.

Kinnberg K. 2003. Evaluation of *in vitro* assays for determination of estrogenic activity in the environment, 57pp. Danish Environmental Protecion Agency, Copenhagen, Denmark.

Kirk L, Tyler C, Lye C, and Sumpter J. 2002. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. Environmental Toxicology and Chemistry 21: 972-979.

KIWA Water Research for GWRC. 2003. Endocrine disrupting compounds – An overview of sources and biological methods for measuring EDC. 16pp.

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

Kojima M, Fukunaga K, Sasaki M., Nakamura M, Tsuji M., and Nishiyama T. 2005. Evaluation of estrogenic activities of pesticides using an in vitro reporter gene assay. International Journal of Environmental Health Research, 15: 271-280.

Kordes C, Rieber EP, and Gutzeit HO. 2002. An in vitro vitellogenin bioassay for oestrogenic substances in the medaka (Oryzias latipes). Aquatic Toxicology 58: 151-164.

Körner W, Bolz U, Süßmuth W, Hiller G, Schuller W, Hanf V, and Hagenmaier H. 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 40: 1131-1142.

Körner W, Hanf V, Schuller W, Kempter C, Metzger J, and Hagenmaier H. 1999. Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. Science of the Total Environment 225: 33-48.

Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg P, and Gustafsson JA. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139: 4252-4263.

Langford KH and Lester JN. 2003. Fate and behaviour of endocrine disrupters in wastewater treatment processes. In Birkett JW and Lester JN (Eds) Endocrine disrupters in wastewater and sludge treatment processes. Lewis Publ. pp. 103-144

Latonnelle K, Le Menn F, Kaushik SJ, and Bennetau-Pelissero C. 2002. Effects of dietary phytoestrogens in vivo and in vitro in rainbow trout and Siberian sturgeon: Interests and limits of the in vitro studies of interspecies differences. General and Comparative Endocrinology 126: 39-51.

Laws SC, Carey SA, Ferrell JM, Bodman GJ, and Cooper RL. 2000. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicological Sciences 54: 154-167.

Laws SC, Stoker TE, Goldman JM, Wilson V, Gray LE, Jr., Cooper, RL. 2005. The US EPA Endocrine Disruptor Screening Program: In Vitro and In Vivo Mammalian Tier I Screening Assays. IN: Developmental and Reproductive Toxicology: A Practical Approach. Ed. RD Hood. Taylor & Francis, New York, NY, USA, pp. 489-524.

Lazier C, Lonergan K, and Mommsen T. 1985. Hepatic estrogen receptors and plasma estrogen-binding activity in the Atlantic salmon. General and Comparative Endocrinology 57: 234-245.

Legler J, Leonards P, Spenkelink A, and Murk A. 2003. *In vitro* biomonitoring in polar extracts of solid phase matrices reveals the presence of unknown compounds with estrogenic activity. Ecotoxicology 12: 239-249.

Legler J, van den Brink C, Brouwer A, Murk A, van der Saag P, Vethaak A, and van der Burg P. 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicological Sciences 48: 55-66.

Legler J, Zeinstra LM, Schuitemaker F, Lanser PH, Bogerd J, Brouwer A, Vethaak AD, De Voogt P, Murk AJ, and Van der Burg B. 2002. Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity. Environmental Science and Technology 36: 4410-4415.

Leusch FDL, Chapman HF, Körner W, Gooneratne SR, and Tremblay LA. 2005. Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environmental Science and Technology 39: 5781-5786.

Leusch FDL, Chapman HF, van den Heuvel MR, Tan BLL, Gooneratne SR, and Tremblay LA. 2006a. Bioassay-derived androgenic and estrogenic activity in municipal sewage in Australia and New Zealand. Ecotoxicology and Environmental Safety doi:10.1016/j.ecoenv.2005.07.020.

Leusch FDL, van den Heuvel MR, Chapman HF, Gooneratne SR, Eriksson AME, and Tremblay LA. 2006b. Development of methods for extraction and in vitro quantification of estrogenic and androgenic activity of wastewater samples. Comparative Biochemistry and Physiology Part C doi:10.1016/j.cpbc.2005.12.010.

Lodish H, Baltimore D, Berk A, Zipursky S, Matsudaira P, and Darnell J. 1995. *Molecular Cell Biology*. Scientific American Books, New York, NY, USA.

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

Louvion JF, Havaux-Copf B, and Picard D. 1993. Fusion of GAL4-VP16 to a steroid-binding domain provides a tool for gratuitous induction of galactose-responsive genes in yeast. Gene 131: 129-134.

Lutz I, and Kloas W. 1999. Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. Science of the Total Environment 225: 49-57.

Matsui S, Takigami H, Matsuda T, Taniguchi N, Adachi J, Kawami H, and Shimizy Y. 2000. Estrogen and estrogen mimics contamination in water and the role of sewage treatment. Water Science and Technology 42: 173-179.

Matsuoka S, Kikuchi M, Kimura S, Kurokawa Y, and Kawai S. 2005. Determination of estrogenic substances in the water of Muko River using in vitro assays, and the degradation of natural estrogens by aquatic bacteria. Journal of Health Science 51: 178-184.

Matthews J, Celius T, Halgren R, and Zacharewski T. 2000. Differential estrogen receptor binding of estrogenic substances: a species comparison. Journal of Steroid Biochemistry and Molecular Biology 74: 223-234.

Matthews J, Fertuck K, Celius T, Huang Y, Fong C, and Zacharewski T. 2002. Ability of structurally diverse natural products and synthetic chemicals to induce gene expression mediated by estrogen receptors from various species. Journal of Steroid Biochemistry and Molecular Biology 82: 181-194.

Matthiessen PM and Gibbs PE. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ. Toxicol, Contam. 17: 37-43.

Metzger DA. 1995. Nontraditional sites of estrogen action. Environmental Health Perspectives 103(S7): 39.

Miller S, Kennedy D, Thomson J, Han F, Smith R, Ing N, Piedrahita J, and Busbee D. 2000. A rapid and sensitive reporter gene that uses green fluorescent protein expression to detect chemicals with estrogenic activity. Toxicological Sciences 55: 69-77.

Munkittrick K, Van Der Kraak G, McMaster M, and Portt C. 1992. Reproductive dysfunction and MFO activity in three species of fish exposed to bleached kraft mill effluent at Jackfish Bay, Lake Superior. Water Pollution Research Journal of Canada 27: 439-446.

Murk A, Legler J, van Lipzig M, Meerman J, Belfroid A, Spenkelink A, van der Burg B, Rijs G, and Vethaak D. 2002. Detection of estrogenic potency in wastewater and surface water with three *in vitro* bioassays. Environmental Toxicology and Chemistry 21: 16-23.

Nakano S, Nagao Y, Kobayashi T, Tanaka M, Hirano S, Nobuhara Y, and Yamada T. 2002. Problems with methods used to screen estrogenic chemicals by yeast two-hybrid assays. Journal of Health Science 48: 83-88.

NIEHS. 2002. Current status of test methods for detecting endocrine disruptors. Expert panel evaluation of the validation status of in vitro test methods for detecting endocrine disruptors. National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA.

Nielsen M, Bøgh IB, Schmidt M, and Greve T. 2001. Immunohistochemical localization of estrogen receptor-alpha in sex ducts and gonads of newborn piglets. Histochemistry and Cell Biology 115(6): 521-526.

Nishihara T, and Nishikawa JI. 2001. Bioassay for endocrine disruptors by using yeast two-hybrid system. Folia Pharmacologica Japonica 118: 203-210.

Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, and Nishihara T. 1999. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicology and Applied Pharmacology 154: 76-83.

OECD. 2001. Detailed Review Paper: Appraisal of Test Methods for Sex Hormone Disrupting Chemicals. OECD Series on Testing and Assessment No 21, 313 pp.

Onda K, Nakamura Y, Takatoh C, Miya A, and Katsu Y. 2003. The behavior of estrogenic substances in the biological treatment process of sewage. Water Science and Technology 47: 109-116.

Parker GJ, Law TL, Lenoch FJ, and Bolger RE. 2000. Development of high throughput screening assays using fluorescence polarization: Nuclear receptor-ligand-binding and kinase/phosphatase assays. Journal of Biomolecular Screening 5: 77-88.

Pawlowski S, Ternes TA, Bonerz M, Rastall AC, Erdinger L, and Braunbeck T. 2004. Estrogenicity of solid phase-extracted water samples from two municipal sewage treatment plant effluents and river Rhine water using the yeast estrogen screen. Toxicology In Vitro 18: 129-138.

Pelletier G, and El-Alfy M. 2000. Immunocytochemical localization of estrogen receptors alpha and beta in the human reproductive organs. Journal of Clinical Endocrinology and Metabolism 85(12): 4835-4840.

Pessala P, Schultz E, Nakari T, Joutti A, and Herve S. 2004. Evaluation of wastewater effluents by small-scale biotests and a fractionation procedure. Ecotoxicology and Environmental Safety 59: 263-272.

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

Pillon A, Boussioux AM, Escande A, Ait-Aissa S, Gomez E, Fenet H, Ruff M, Moras D, Vignon F, Duchesne MJ, Casellas C, Nicolas JC, and Balaguer P. 2005. Binding of estrogenic compounds to recombinant estrogen receptor-alpha: Application to environmental analysis. Environmental Health Perspectives 113: 278-284.

Purdom C, Hardiman P, Bye V, Eno N, Tyler C, and Sumpter J. 1994. Estrogenic effects of effluents from sewage treatment works. Chemistry and Ecology 8: 275-285.

Quabius ES, Nolan DT, Allin CJ, and Wendelaar-Bonga SE. 2000. Influence of dietary exposure to polychlorinated biphenyl 126 and nutritional state on stress response in tilapia (*Oreochoromis mossambicus*) and rainbow trout (*Oncorhyncus mykiss*). Environmental Toxicology and Chemistry 19: 2892-2899.

Rasmussen TH, and Nielsen JB. 2002. Critical parameters in the MCF-7 cell proliferation bioassay (E-Screen). Biomarkers 7: 322-336.

Rehmann K, Schramm K, and Kettrup A. 1999. Applicability of a yeast oestrogen screen for the detection of oestrogen-like activities in environmental samples. Chemosphere 38: 3303-3312.

Robinson CD, Brown E, Craft JA, Davies IM, Moffat CF, Pirie D, Robertson F, Stagg RM, and Struthers S. 2003. Effects of sewage effluent and ethynyl oestradiol upon molecular markers of oestrogenic exposure, maturation and reproductive success in the sand goby (Pomatoschistus minutus, Pallas). Aquatic Toxicology 62: 119-134.

Rogers JM, and Denison MS. 2000. Recombinant cell bioassays for endocrine disruptors: Development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. In Vitro & Molecular Toxicology 13: 67-82.

Rosenberg Zand RS, Jenkins DJA, and Diamandis EP. 1999. Development and evaluation of a competitive time-resolved immunofluorometric assay for the estrogen-regulated protein pS2. Journal of Clinical Laboratory Analysis 13: 241-245.

Routledge E, Sheahan D, Desbrow C, Brighty G, Waldock M, and Sumpter J. 1998. Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. Environmental Science and Technology 32: 1559-1565.

Routledge E, and Sumpter J. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry 15: 241-248.

Rutishauser B, Pesonen M, Escher B, Ackermann G, Aerni H, Suter M, and Eggen R. 2004. Comparative analysis of estrogenic activity in sewage treatment plant effluents involving three in vitro assays and chemical analysis of steroids. Environmental Toxicology and Chemistry 23: 857-864.

Saito M, Tanaka H, Takahashi A, and Yakou Y. 2002. Comparison of yeast-based estrogen receptor assays. Water Science and Technology 46: 349-354.

Sarmah AK, Northcott GL, Leusch FDL, and Tremblay LA. 2006. A survey of endocrine disrupting chemicals (EDCs) in municipal sewage and animal waste effluents in the Waikato region of New Zealand. Science of the Total Environment 335(1-3): 98-105.

Schiliro T, Pignata C, Fea E, and Gilli G. 2004. Toxicity and estrogenic activity of a wastewater treatment plant in Northern Italy. Archives of Environmental Contamination and Toxicology 47: 456-462.

Schultis T, and Metzger JW. 2004. Determination of estrogenic activity by LYES-assay (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase). Chemosphere 57: 1649-1655.

Seifert M. 2004. Luminescent enzyme-linked receptor assay for estrogenic compounds. Analytical and Bioanalytical Chemistry 378: 684-687.

Seifert M, Haindl M, and Hock B. 1999. Development of an enzyme linked receptor assay (ELRA) for estrogens and xenoestrogens. Analytica Chimica Acta 386: 191-199.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren J, Bokesch H, Kenney S, and Boyd M. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute 82: 1107-1112.

Snyder S, Villeneuve D, Snyder E, and Giesy J. 2001. Identification and quantification of estrogen receptor agonists in wastewater effluents. Environmental Science and Technology 35: 3620-3625.

Sohoni P, and Sumpter J. 1998. Several environmental oestrogens are also anti-androgens. Journal of Endocrinology 158: 327-339.

Soto A, Sonnenschein C, Chung K, Fernandez M, Olea N, and Olea-Serrano M. 1995. The E-Screen assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental Health Perspectives 103: 113-122.

Soto AM, Calabro JM, Prechtl NV, Yau AY, Orlando EF, Daxenberger A, Kolok AS, Guillette LJ, le Bizec B, Lange IG, and Sonnenschein C. 2004. Androgenic and estrogenic activity in

water bodies receiving cattle feedlot effluent in eastern Nebraska, USA. Environmental Health Perspectives 112: 346-352.

Sumida K, Ooe N, Nagahori H, Saito K, Isobe N, Kaneko H, and Nakatsuka I. 2001. An *in vitro* reporter gene assay method incorporating metabolic activation with human and rat S9 or liver microsomes. Biochemical and Biophysical Research Communications 280: 85-91.

Sumpter J. 1995. Feminized responses in fish to environmental estrogens. Toxicology Letters 82/83: 737-742.

Ternes T, Kreckel P, and Mueller J. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants - II. Aerobic batch experiments with activated sludge. Science of the Total Environment 225: 91-99.

Thomas K, Hurst M, Matthiessen P, and Waldock M. 2001. Characterization of estrogenic compounds in water samples collected from United Kingdom estuaries. Environmental Toxicology and Chemistry 20: 2165-2170.

Thomas P. 2003. Rapid, nongenomic steroid actions initiated at the cell surface: lessons from studies with fish. Fish Physiology and Biochemistry 28: 3-12.

Tilton F, Benson WH, and Schlenk D. 2002. Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. Aquatic Toxicology 61: 211-224.

Tollefsen KE, Mathisen R, and Stenersen J. 2003. Induction of vitellogenin synthesis in an Atlantic salmon (Salmo salar) hepatocyte culture: a sensitive in vitro bioassay for the oestrogenic and anti-oestrogenic activity of chemicals. Biomarkers 8: 394-407.

Toomey B, Monteverdi G, and Di Giulio R. 1999. Octylphenol induces vitellogenin production and cell death in fish hepatocytes. Environmental Toxicology and Chemistry 18: 734-739.

Tremblay L, and Van Der Kraak G. 1998. Use of a series of homologous in vitro and in vivo assays to evaluate the endocrine modulating actions of beta-sitosterol in rainbow trout. Aquatic Toxicology 43: 149-162.

Tully DB, Cox VT, Mumtaz MM, Davis VL, and Chapin RE. 2000. Six high-priority organochlorine pesticides, either singly or in combination, are nonestrogenic in transfected HeLa cells. Reproductive Toxicology 14: 95-102.

U.S. EPA. 2000. U.S. Environmental Protection Agency – Endocrine Disruptor Screening Program. Report to Congress, U.S. EPA.

Vethaak AD, Rijs GBJ, Schrap SM, Ruiter H, Gerritsen A, and Lahr J. 2002. Estrogens and xeno-estrogens in the aquatic environment of The Netherlands: Occurrence, potency and biological effects . RIZA/RIKZ report No. 2002.001. Lelystad, The Netherlands.

Villalobos M, Olea N, Brotons J, Olea-Serrano M, de Almodovar J, and Pedraza V. 1995. The E-Screen assay: a comparison of different MCF7 cell stocks. Environmental Health Perspectives 103: 844-850.

Vinggaard A, Joergensen E, and Larsen J. 1999. Rapid and sensitive reporter gene assays for detection of antiandrogenic and estrogenic effects of environmental chemicals. Toxicology and Applied Pharmacology 155: 150-160.

Vonier P, Crain D, McLachlan J, Guillette LJ, and Arnold S. 1996. Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American Alligator. Environmental Health Perspectives 104: 1318-1322.

Willingham E. 2001. Embryonic exposure to low-dose pesticides: Effects on growth rate in the hatchling red-eared slider turtle. Journal of Toxicology and Environmental Health Part A 64: 257-272.

Wilson VS, Bobseine K, and Gray LE. 2004. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicological Sciences 81: 69-77.

Witters H, Vangenechten C, and Berckmans P. 2001. Detection of estrogenic activity in Flemish surface waters using an *in vitro* recombinant assay with yeast cells. Water Science and Technology 43: 117-123.

Yadetie F, Arukwe A, Goksøyr A, and Male R. 1999. Induction of hepatic estrogen receptor in juvenile Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. Science of the Total Environment 233: 201-210.

Yoon K, Pellaroni L, Ramamoorthy K, Gaido K, and Safe S. 2000. Ligand structure-dependent differences in activation of estrogen receptor alpha in human HepG2 liver and U2 osteogenic cancer cell lines. Molecular and Cellular Endocrinology 162: 209-219.

Zacharewski T. 1997. *In vitro* bioassays for assessing estrogenic substances. Environmental Science and Technology 31: 613-623.

Zacharewski T. 1998. Identification and assessment of endocrine disruptors: Limitations of *in vivo* and *in vitro* assays. Environmental Health Perspectives 106: 577-582.

Zacharewski T, Berhane K, Gillesby B, and Burnison B. 1995. Detection of estrogen- and dioxin-like activity in pulp and paper mill black liquor and effluent using *in vitro* recombinant receptor/reporter gene assays. Environmental Science and Technology 29: 2140-2146.

Appendix I

Summary of *in vitro* Assays

Table 1. Summary of Main Characteristics of *in vitro* Bioassays Covered in this Review.

ID	Name	Page	Class [1]	Reference	Estimated DL [2]	Use	Duration	Pros	Cons	Env. Ref. [3]
01	Standard ER binding assay (ERBA)	18	RBA	ICCVAM 2003	0.3 – 14 ng/L EC50 = 0.1 - 2.5 nM (but depends on the concentration of radioligand) DF = 10 – 20x	+++	< 24 h	- Simple - Rapid - Measures both agonists and antagonists - ER can be isolated or purchased - Low matrix interference	- Poor predictor of whole-organism response - Small quantities of radioactive waste	Leusch et al. 2005; Leusch et al. 2006a; Lutz and Kloas 1999; Murk et al. 2002; Pillon et al. 2005; Sarmah et al. 2006
02	Fluorescence polarization ER binding assay (FP-ERBA)	18	RBA	Parker et al. 2000	2.5 – 40 ng/L EC50 = 5 – 15 nM (but depends on the concentration of fluormone) DF = 2 – 10x	++	< 12 h	- Very quick - Measures both agonists and antagonists - Available as a commercial kit	- Poor predictor of whole-organism response - Medium matrix interference (background fluorescence) - Requires specific equipment for FP	Conroy et al. 2005
03	Enzyme-linked receptor assay (ELRA)	19	RBA	Seifert 2004	0.1 – 0.6 ng/L EC50 = 0.2 – 0.5 nM DF = 2 – 4x	-	< 12 h	- Very quick - Very low DL - Measures both agonists and antagonists	- Recent technique, not easily available - Poor predictor of whole-organism response	Seifert 2004; Seifert et al. 1999

ID	Name	Page	Class [1]	Reference	Estimated DL [2]	Use	Duration	Pros	Cons	Env. Ref.
04	Yeast estrogen screen (YES)	21	yRGA	Routledge and Sumpter 1996; De Boever et al. 2001	0.6 – 2.7 ng/L EC50 = 0.1 – 0.5 nM DF = 20x	+++	2-3 d	- Simple assay - Endpoint measured by spectrophotometer - Widely available	- Poor predictor of whole-organism response - Medium matrix interference (cytotoxicity)	Multiple references, including Brooks et al. 2003; Conroy et al. 2005; Desbrow et al. 1998; Kirk et al. 2002; Matsui et al. 2000; Matsuoka et al. 2005; Murk et al. 2002; Onda et al. 2003; Pawlowski et al. 2004; Rutishauser et al. 2004; Thomas et al. 2001; Tilton et al. 2002; Witters et al. 2001
05	Yeast-based reporter gene assay	23	yRGA	Gaido et al. 1997	25 – 100 ng/L EC50 = 0.1 – 0.4 nM DF = 1,000x	++	1-2 d	- Endpoint measured by spectrophotometer	- Poor predictor of whole-organism response - Work-intensive - High matrix interference (cytotoxicity)	
06	Yeast two-hybrid assay	23	yRGA	Nishikawa et al. 1999	5 – 10 ng/L EC50 = 1 – 2 nM DF = 20x	+	< 24 h	- Endpoint measured by spectrophotometer - Relatively rapid - Analysis of molecular interactions	- Poor predictor of whole-organism response - High matrix interference (cytotoxicity)	Fukazawa et al. 2002

ID	Name	Page	Class [1]	Reference	Estimated DL [2]	Use	Duration	Pros	Cons	Env. Ref.
07	Hybrid receptor yeast-based assay	24	yRGA	Louvion et al. 1993	25 – 50 ng/L EC50 = 1 – 2 nM DF = 100x	-	< 24 h	- Endpoint measured by spectrophotometer - Relatively rapid	- Poor predictor of whole-organism response - High matrix interference (cytotoxicity) - Assay performed in Erlenmeyer flasks	Rehmann et al. 1999
08	Yeast enhanced green fluorescent protein (yEGFP)	25	yRGA	Bovee et al. 2004	5 – 30 ng/L EC50 = 0.2 – 1 nM DF = 100x	-	< 24 h	- Relatively rapid - Well adapted for high-throughput screening and automation	- Medium matrix interference (cytotoxicity) - Poor predictor of whole-organism response	
09	MVLN	25	vRGA	Demirpence et al. 1993	0.25 – 1.3 ng/L EC50 = 0.01 – 0.05 nM DF = 100x	++	4-5 d		- Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	Coors et al. 2004; Furuichi et al. 2004; Itoh et al. 2000; Snyder et al. 2001
10	MELN	26	vRGA	Balaguer et al. 1999	0.15 – 0.4 ng/L EC50 = 0.005 – 0.015 nM DF = 100x	++	2-3 d	- Relatively rapid	- Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	Balaguer et al. 2000; Cargouet et al. 2004; Fenet et al. 2003; Pillon et al. 2005

ID	Name	Page	Class [1]	Reference	Estimated DL [2]	Use	Duration	Pros	Cons	Env. Ref.
11	ER-CALUX	26	vRGA	Legler et al. 1999	0.7 – 1.3 ng/L EC50 = 0.005 – 0.01 nM DF = 500x	+++	2-3 d	- Relatively rapid - Uses T47D breast cancer cells which endogenously express both ERα and ERβ	- Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity) - Patent-related costs	Legler et al. 2003; Murk et al. 2002; Vethaak et al. 2002
12	T47D-Kbluc	26	vRGA	Wilson et al. 2004	0.4 - 1.3 ng/L EC50 = 0.00.3 - 0.01 nM DF = 500x	+	2 d	- Relatively rapid - Cells easily available - Good level of technical support - Uses T47D breast cancer cells which endogenously express both ERα and ERβ	- Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	
13	BG1Luc4E2	26	vRGA	Rogers and Denison 2000	0.15 – 0.4 ng/L EC50= 0.005 – 0.015 nM DF = 100x	+	2 d	- Relatively rapid	- Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	
14	HELNa and HELNb	27	vRGA	Balaguer et al. 1999	0.15 – 0.4 ng/L EC50= 0.005 – 0.015 nM DF = 100x	-	2-3 d	- Allows determination of effects on different isomers of the ER	- Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	

ID	Name	Page	Class [1]	Reference	Estimated DL [2]	Use	Duration	Pros	Cons	Env. Ref.
15	HGELN	27	vRGA	Balaguer et al. 1999	0.5 – 2 ng/L EC50 = 0.02 – 0.08 nM DF = 100x	+	4 d	- Very specific estrogenic response	- Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	
16	MCF7-ERE-GFP	28	vRGA	Miller et al., 2000	2.7 – 14 ng/L EC50 = 0.01 – 0.05 nM DF = 1,000x	-	1-2 d	- Relatively quick	- Requires PC2 facilities - Requires high level of expertise - Time consuming - Low-throughput in its current form - Medium matrix interference (cytotoxicity)	
17	E2 Bioassay	28	vRGA	Zacharewski et al. 1995	2.7 – 14 ng/L EC50 = 0.01 – 0.05 nM DF = 1,000x	+	2 d	- Very specific estrogenic response	- Transient transfection - Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	Zacharewski et al. 1995

ID	Name	Page	Class [1]	Reference	Estimated DL [2]	Use	Duration	Pros	Cons	Env. Ref.
18	RTG-2 cell bioassay	29	vRGA	Fent 2001	5 – 10 ng/L EC50 = 0.2 – 0.4 nM DF = 100x	-	3 d	- Based on fish cell line	- Transient transfection - Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	Rutishauser et al. 2004
19	Ishikawa cell ALP	30	ivGEA	Matsuoka et al. 2005	0.5 – 5 ng/L EC50 = 0.01 – 0.1 nM DF = 200x	-	4 d	- Natural estrogenic response - Endpoint measured by spectrophotometer	- Specificity of ALP induction?	Matsuoka et al. 2005
20	BT-474 pS2 assay	30	ivGEA	Rosenberg Zand et al. 1999	0.5 – 3 ng/L EC50 = 0.02 – 0.05 nM DF = 100 – 200x	-	8 d	- Natural estrogenic response	- Relatively long exposure required - Specificity of pS2 induction?	
21	HEP-Vtg	30	ivGEA	Tollefsen et al. 2003	25 – 130 ng/L EC50 = 0.1 – 0.5 nM DF = 1,000x	+++	5 d	- Natural estrogenic response - Endpoint easily measured by ELISA	- Requires availability of source organism (eg fish)	Vethaak et al. 2002
22	HEP-Zrp	30	ivGEA	Rutishauser et al. 2004	40 ng/L EC50 = 1 nM DF = 150x	-	4 d	- Natural estrogenic response - Endpoint measured by ELISA	- Requires availability of source organism (eg fish)	Rutishauser et al. 2004

ID	Name	Page	Class [1]	Reference	Estimated DL [2]	Use	Duration	Pros	Cons	Env. Ref.
23	E-Screen	32	CPA	Körner et al. 1999; Soto et al. 1995	0.3 – 3 ng/L EC50 = 0.005 – 0.01 nM DF = 200 – 1,000x	+++	5-7 d	- Widely used - Endpoint measured by spectrophotometer	- Relatively long exposure duration - High variability - Specificity of the response? - Requires PC2 facilities - Requires high level of expertise - Medium matrix interference (cytotoxicity)	Drewes et al. 2005; Körner et al. 2000; Körner et al. 1999; Leusch et al. 2005; Matsuoka et al. 2005; Schiliro et al. 2004; Soto et al. 2004
24	Yeast PL3 growth test	32	CPA	Connor et al. 1996	16 – 80 ng/L EC50 = 3 nM DF = 20 – 100x	-	4-6 d	- Endpoint easily measured (no. of yeast colonies)	- Work intensive - Specificity of the response? - Requires PC2 facilities - Requires high level of expertise	

[1] Different classes are: Receptor Binding Assay (RBA), yeast Reporter Gene Assay (yRGA), vertebrate Reporter Gene Assay (vRGA), *In vitro* Gene Expression Assay (ivGEA), and Cell Proliferation Assay (CPA).
[2] Estimated DL based on a 1000-fold sample concentration through solid-phase extraction, calculated as the product of the EC50 and the dilution factor.
[3] Relevant environmental references where that type of assay has been used to determine estrogenicity of environmental water samples.

Appendix II

In vivo Bioassays

In vivo bioassays rely on the concept of biomarkers. Biomarkers are measurable morphological, physiological, or behavioural responses known to be induced by exposure to estrogen or estrogen-mimics (such as increased plasma Vtg levels in male fish exposed to estrogens). In *in vitro* tests, parts (cells or organs) of whole organisms are used. While this approach allows researchers to measure effects-based estrogenicity without the ethical cost of maintained animal experimentation, organisms are more complicated than just the sum of their individual parts. Complex hormonal (endocrine) communication between different cells and organs within the body mean that effects on the endocrine system cannot be wholly explained through *in vitro* analysis. In *vivo* assays are more expensive and time-consuming than *in vitro* bioassays, but incorporate important biological processes that are limited in *in vitro* bioassays, such as metabolic biotransformation and active transport mechanisms. Also, while *in vitro* bioassays are mostly limited to ER-mediated effects, *in vivo* assays also evaluate estrogenic effects via non ER-mediated pathways (such as interference with steroid and receptor biosynthesis) and integrate the robustness of the organism to estrogenic modulation (due in part to complex feedback mechanisms involved in endocrine homeostasis). Because of this array of feedback systems in endocrine communication, estrogenic effects in many *in vivo* bioassays can also be linked to antiandrogenic effects, and it is often impossible to differentiate between the two.

The following provides an overview of some of the currently developed *in vivo* bioassays used to quantify estrogenicity in water samples.

A.1 Fish

Several endpoints can be used as indicators of endocrine disruption in fish. The most widely used biomarker of exposure to estrogenic chemicals in male fish is Vtg induction (Denslow et al. 1999) (Note that Vtg induction in females can also be used as an indicator of exposure to estrogenicity, although it is not as sensitive as in male fish). Levels of Vtg can be measured using enzyme-linked immunosorbent assay (ELISA) in many different species of fish (Ataria et al. 2004; Folmar et al., 2000; Holbech et al., 2001; Korsgaard and Pedersen, 1998; Lomax et al., 1998; Pait and Nelson, 2003; Sherry et al., 1999). Vitellogenin induction can also be quantified by measuring the levels of Vtg mRNA (Bowman and Denslow,1999; Denslow et al., 2001a; Islinger et al., 2002; Lattier et al., 2001). Induction of specific genes can also be used to determine if animals have been exposed to estrogenic chemicals, and can be measured by novel techniques such as microarrays (Larkin et al., 2002), differential display reverse transcription polymerase chain reaction (RTPCR) (Denslow et al., 2001b), and real time RTPCR (Leusch et al., 2005). Morphological endpoints can also be used to determine exposure to endocrine disruptors. Ovotestis (the presence of both oocytes and testicular tissue in gonads of the same individual) has been used as an indicator of exposure to EDCs (Bortone and Davis, 1994; Jobling et al., 1998), with a well described histopathology (Nolan et al. 2001), although this endpoint is specific to estrogenic EDCs. Development of secondary sexual characteristics in fish is mostly directed by androgens and estrogens (Bond, 1996; Hadley, 1988), and abnormal

development of secondary sexual characteristics in mosquitofish (*Gambusia affinis and G. holbrooki*) has been used as an indicator of exposure to chemicals with estrogenic activity (Denton et al.,1985; Doyle and Lim 2002; Drèze et al., 2000). Finally, circulating levels of plasma steroids and relative gonad size (GSI) have also been measured in fish exposed sewage effluent (Angus et al., 2002; Folmar et al., 2001; Jobling et al., 2002) and provide a direct assessment of their endocrine status.

Fish are excellent models to study the effects of EDCs in water samples, being exposed to chemicals via both dietary and waterborne pathways (Kime, 1999). Fish are also clearly sensitive to exposure to exogenous steroids, a fact that has long been used in aquaculture to direct sex determination (Baroiller and D'Cotta, 2001; Piferrer, 2001).

A.1.1 Gene Induction

Vitellogenin gene induction

Vitellogenin (Vtg) is a glycolipophosphoprotein precursor to egg yolk produced in the liver of mature female fish under estrogenic stimulation. Although it is only detected at very low levels in males under normal conditions, Vtg expression can be greatly induced in males exposed to exogenous estrogens (Denslow et al., 1999). This abnormally high production of a female-specific protein in male fish has been extensively used as a sensitive biochemical indicator of exposure to estrogenic chemicals in many fish species (Folmar et al., 1996; Harries et al., 1996; Nakari, 2004; Porter and Janz, 2003).

There are many examples of this assay in the literature, using several different species of fish and different endpoints. The following protocol is a generalisation.
Immature or adult male fish are exposed to the water samples (whether in laboratory tanks or caged in the field) for several days. The fish are then sampled, and Vtg protein or mRNA levels measured. The exposure duration depends on the endpoint used, with induction of Vtg mRNA usually detectable within 4-7 d and increase in plasma Vtg concentrations detectable within 7-21 d.

Vtg protein in the plasma or in whole body homogenates (in the case of smaller fish species) can be quantified with a variety of methods. Sampling plasma is a very ethically-viable option, as fish can be released (in case of field sampling), or sampled again at a later date in the case of caging or laboratory exposures (this however only applies to large fish species which can be sampled non-destructively).

- **ELISA** (Denslow et al., 1999): Probably the most commonly used method to measure Vtg has been with enzyme-linked immunosorbent assay (ELISA). ELISA are very sensitive, and have been developed for a wide range of fish species, including (amongst many others) Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and fathead minnows (*Pimephales promelas*) (Nilsen et al., 2004), and a generalized Vtg ELISA may be used for most species (Heppell et al., 1995).
- **RIA** (Tyler and Sumpter 1990): Radioimmunoassays (RIA) are another very sensitive method to measure Vtg protein, and has been used mostly in the United Kingdom to

measure plasma Vtg in carp (*Cyprinus carpio*) (Tyler and Sumpter 1990), rainbow trout (*Oncorhynchus mykiss*) (Purdom et al., 1994), roach (*Rutilus rutilus*) (Routledge et al., 1998), and fathead minnows (Panter et al., 2000). This method is as sensitive as the ELISA method, but produces small quantities of radioactive waste and requires appropriately licensed laboratory facilities.

- **Immunoblotting:** Immunoblotting (western blot) is not as sensitive as the other methods described above and may be less adequate for quantitation, but offers the advantage of high specificity and unequivocally identifying the protein (Petrovic et al., 2002). It is more often used in research and validation, while the other methods may be more suitable to environmental monitoring.
- **Indirect methods** (Verslycke et al. 2002): Plasma Vtg can also be measured indirectly by measuring plasma alkaline-labile phosphorous (ALP) (Kramer et al., 1998) or plasma calcium levels (Verslycke et al., 2002). Vtg is the only phosphorous-containing protein in the blood of oviparous vertebrates, and the presence of ALP in fish plasma is therefore highly correlated with Vtg levels. Each protein phosphate group in the Vtg protein is associated with a calcium atom, and plasma calcium can therefore also be an indirect measure of Vtg levels. Both plasma ALP and plasma calcium are highly correlated with plasma Vtg, and may be easy and inexpensive alternatives with similar sensitivity (Verslycke et al., 2002).

Alternatively, Vtg expression can me measured by quantifying Vtg mRNA levels in livers or whole body homogenates. Measuring mRNA levels usually require more expensive equipment and consumables than are used for measuring protein levels.

- **HPA** (Thomas-Jones et al., 2003): In a hybridisation protection assay (HPA), a specific chemiluminescent DNA probe is added to samples of total RNA (prepared from whole body homogenates), and the DNA probe hybridises to its target (if present). An alkaline reagent is added which selectively degrades unhybridised probes rapidly. Chemiluminescence (from the undegraded hybridised probes) is then measured. This technique has currently only been developed for fathead minnows, but can be adapted to any species where the Vtg RNA sequence is known.
- **Real time RTPCR** (Leusch et al., 2005): In this real time reverse transcription polymerase chain reaction (real time RTPCR) procedure, total RNA (isolated from livers) is reverse transcribed into cDNA, which is then amplified in a real time PCR reaction (in a one-step reaction). The LUX™ (Invitrogen) DNA primers used in this reaction fluoresce when unfolded and incorporated into a cDNA amplicon. The number of cDNA amplicons is determined by fluorescence for each cycle of the PCR reaction, and Vtg mRNA induction (relative to a house-keeping gene, 18S rRNA) can be quantified relative to an estradiol standard curve or a reference site. At the moment, this method has only been used in mosquitofish (*Gambusia affinis* and *G. holbrooki*), but can be adapted to any species where the Vtg RNA sequence is known.
- **Array technology** (Alberti et al., 2005): In this procedure (also known as reverse Southern blotting), extracted liver and gonadal mRNA is reverse transcribed into fluorescently labelled cDNA, and applied to an array chip containing DNA probes for specific genes. After a short incubation to allow for hybridisation, the array is rinsed (to remove unbound DNA) and fluorescence in each gene spot is measured by an array scanner. The array scan is then compared with that of a control fish to determine, on a

gene by gene basis, if it is brighter (over-expressed) or darker (under-expressed) in the test fish compared with the control. Gene arrays can therefore be used to measure gene expression of many different genes (known as gene expression profiling), including estrogen-mediated genes such as Vtg. This paper describes the method specifically for zebrafish, but arrays can be developed for any species where estrogen-responsive genes (such as Vtg) have been sequenced, and a similar technique is also already in use with sheepshead minnows (*Cyprinodon variegates*) (Larkin et al. 2002). Gene array technology is still very recent and does not currently allow absolute quantification of mRNA levels (induction and inhibition are always expressed relative to another sample), but has the potential to be a very powerful tool in ecotoxicology.

- **Other methods:** Liver Vtg mRNA can also be quantified using Northern blots and equivalents (Folmar et al., 2000), RTPCR followed by Southern blot (Bowman and Denslow 1999), or differential display RTPCR (Denslow et al., 2001a). However, accurate quantification in these techniques is limited by the intrinsic inaccuracy of photo-imaging density analysis, and blot-based techniques may be more suitable to research situations.

A significant increase in Vtg can be measured at roughly 10-100 ng/L E2 after 7-21 d exposure, but depends on the sensitivity of each fish species and the endpoint measured. For example, significant induction of Vtg mRNA occurred after just 2 d of exposure to 100 ng/L of E2 in sheepshead minnows (Denslow et al. 2001a), after 4 d of exposure to 250 ng/L of E2 in adult male mosquitofish (Leusch et al., 2005), and after 7 d of exposure to 25 ng/L of the more potent synthetic estrogen ethynylestradiol (EE2) in male Japanese medaka (Islinger et al., 2002). A significant increase in plasma Vtg was detected after 21 d of exposure to10 ng/L of E2 in rainbow trout and roach (Routledge et al., 1998), and after 14 d of exposure to 1 ng/L to EE2 in rainbow trout (Thorpe et al., 2003). A significant increase in whole body homogenate Vtg occurred after 7 d of exposure to 10 ng/L of EE2 in adult male zebrafish (Holbech et al. 2001), and a significant increase in plasma calcium and plasma ALP was detectable after 14 d of exposure to 100 ng/L of E2 in rainbow trout (Verslycke et al., 2002).

This method is very flexible, and fish can be exposed either to water samples in the laboratory or caged in the field to provide a measure of estrogenicity of the water. Vtg induction is considered by many to be the golden standard of estrogenicity in water.

Vitellogenin gene induction can also be done *ex-vivo*, using liver slices (Shilling and Williams 2000). This assay is similar to the fish HEP-Vtg (assay no. 20, p. 24) except that it uses whole liver slices instead of hepatocytes, and is thus more representative of the organ *in vivo* and preserves cell heterogeneity and cell-to-cell interactions. Liver slices are incubated in culture medium containing the samples for 96 h on orbital shakers. At the end of the exposure period, livers are homogenized in phosphate buffer and Vtg quantified by ELISA.

Culture conditions are discussed in Schmieder et al. (2000). Although this assay is generally done with rainbow trout liver slices, it has also been done with frog (*Xenopus laevis*) liver slices with a similar sensitivity (Hurter et al., 2002).

Zrp induction assay (Arukwe et al., 1997)

These tests use the same approach as previously described for the Vtg induction assay, but measure Zrp protein or mRNA levels as indicators of exposure. As in the Vtg induction assay, fish are exposed for several days (usually 7-21 d) and the level of plasma Zrp or liver Zrp mRNA is measured.

It is difficult to determine how sensitive Zrp induction is, as experiments measuring Zrp in fish so far have focused mostly on intraperitoneal injections instead of waterborne exposures, although it appears to be even more sensitive than Vtg induction in Atlantic salmon after exposure to estrogenic chemicals (Arukwe et al., 1997; Yadetie et al., 1999). Robinson et al. (2003) report significantly increased Zrp mRNA levels in both male and female sand gody (*Pomatoschistus minutus*) exposed for 6 weeks to 6 ng/L of EE2. Zrp may thus be an excellent alternate biomarker of estrogenicity.

ER induction assay (MacKay et al., 1996)

The ER gene is also under estrogen-control, and exposure to estrogenic chemicals have been shown to increase the levels of liver ER mRNA and cellular ER in *O. mykiss*. Thus, liver ER mRNA and cellular ER has been used as a biomarker of exposure to estrogenic chemicals, in a manner similar to that described previously for Vtg and Zrp. Immature fish are exposed to the sample for several days (usually 7-21 d). After exposure, livers are excised. Hepatic ER mRNA can then be measured using the techniques described above (RTPCR, Northern and slot blotting), while ER can be measured by western blot or by performing an ER binding assay with the liver extracts (as described in Section 2.0 of this report).

The sensitivity of ER induction assay is also difficult to determine to due to the lack of waterborne dose-response data, but probably slightly lower than those of Zrp and Vtg based on quicker response of ER mRNA after exposure to the estrogenic chemical nonyphenol (Yadetie et al., 1999). ER mRNA levels appear not to be well correlated with ER levels, apparently due to post-transcriptional regulation mechanisms (MacKay et al., 1996). ER mRNA induction also appears to return to pre-exposure levels relatively quickly (within 3-4 d) after an initial peak (Flouriot et al., 1997; Yadetie et al., 1999), probably because of self-regulating feedback systems, and determination of the ER protein seems a more reliable endpoint. Indeed, injecting immature rainbow trout with E2 for 2-3 weeks is a common procedure to increase the yield of ER prior to isolating hepatic ER for receptor binding assays (Tremblay and Van Der Kraak, 1998).

A.1.2 Transgenic Fish

Transgenic luminescent zebrafish (Legler et al., 2000)

This transgenic zebrafish bioassay (Legler et al., 2000) is basically a larger scale ER-CALUX assay, using whole zebrafish instead of breast cancer cells. Zebrafish embryos are microinjected with a luciferase reporter gene regulated by an ERE linked to a TATA box (pEREtata-Luc). When exposed to estrogenic chemicals, the cells of the zebrafish will produce luciferase, which can then be measured by luminescence.

Zebrafish embryos are injected with the luciferase reporter gene, and successfully transfected fish are selected and bred. Adult F2 and juvenile F3 fish are then exposed to water samples for 48-96 h. They are sacrificed and dissected, and individual organs are homogenized prior to luciferase activity assay. This method also allows detailed immunohistochemistry (using anti-luciferase antibodies) studies of estrogen-responsive organs.

Liver and testis are the most responsive organs, and 96 h exposure of adult transgenic zebrafish to 300 ng/L of E2 results in significantly increased luciferase activity in testis.

Transgenic fluorescent zebrafish (Hsiao and Tsai, 2003)

In this assay, transgenic zebrafish germ cells are harbouring an enhanced green fluorescent protein (EGFP) gene driven by a medaka β-actin promoter. Juvenile zebrafish are hermaphroditic, with undifferentiated gonads first developing into ovary-like tissues, which then either become ovaries in fully developed females or degenerate and develop into testes in males. In this assay, proliferating germs cells and female gonads strongly express EGFP, but fluorescence is only dimly detected in males. This technique allows the study of germ cells, which has been shown to be sensitive to environmental pollutants (Van den Belt et al., 2001). Although promising, this technique has not been used in the study of environmental EDCs and it is unclear how specific or sensitive to exposure to estrogenic chemicals germ cell development in zebrafish is.

Transgenic medaka (Ueno et al., 2004)

A transgenic medaka with a green fluorescent protein (GFP) gene linked to an estrogen-dependent gene (in this case choriogenin, a precursor protein of egg envelope in medaka). Liver GFP expression is thus dependent on E2 exposure. Significant fluorescence can be detected in E2-exposed males within 2-3d exposure to 10ng/mL at varying stages of development.

A.1.3 Sexual Abnormalities

Gonadal sex ratio and intersex

Sex determination in certain species of fish can exhibit a fair level of plasticity, and can be controlled by external environmental factors such as temperature or exposure to hormonally-active chemicals (Baroiller and D'Cotta, 2001; Piferrer, 2001). Sex ratio in fish that exhibit environmentally-determined sex can therefore provide an indication of estrogenic potency of the water sample. Intersexuality (the presence of both male and female sex cells in gonads, also called ovo-testis or testis-ova) was the first indicator of endocrine disruption in wild fish exposed to treated sewage in the United Kingdom (Purdom et al., 1994), and the presence of intersex individuals in gonochoristic species has since been used as a classical indicator of endocrine disruption (Bortone and Davis, 1994) in several species of fish, including roach (Jobling et al., 1998), Japanese medaka (Hartley et al., 1998), zebrafish (Andersen et al., 2003), bream (*Abramis brama*) (Vethaak et al., 2005), gudgeon (*Gobio gobio*) (van Aerle et al., 2001), and catfish

(*Clarias gariepinus*) (Barnhoorn et al., 2004). Feminization of the reproductive ducts has also been used to identify estrogenicity in water in roach (Rodgers-Gray et al., 2001) and fathead minnows (van Aerle et al., 2002).

Eggs are exposed to the samples from fertilisation or immediately post-hatch until they are fully developed, which can take from 60-100 d in small fish species such as *D. rerio* (Andersen et al.,. 2003) and *O. latipes* (Metcalfe et al., 2001). The fish are then sexed histologically, and a higher proportion of females (or intersex individuals) in exposed vs control groups is an indicator of estrogenicity.

The sensitivity of this bioassay depends on the species, but has been shown to be relatively sensitive, with a significant bias towards female (as well as intersex) fish in *O. latipes* exposed to 10 ng/L E2 (Metcalfe et al., 2001) and complete feminization of *D. rerio* after exposure to only 15 ng/L EE2 (Andersen et al., 2003).

Morphological changes of the gonad (such as intersex) are permanent, and thus significantly more deleterious than for example increased plasma Vtg, which has been shown to return to normal levels after depuration (Van den Belt et al., 2002). However such changes can generally only be induced in early developmental stages, and intersex cannot be induced in fully mature individuals (van Aerle et al., 2002), while Vtg can be induced both in immature and mature individuals.

Secondary sexual characteristics in poeciliids

Inhibition of secondary sexual characteristics has been a widely used biomarker of exposure to hormonally-active chemicals in sexually dimorphic poeciliid fish, such as mosquitofish (*Gambusia*). Male mosquitofish are much smaller than the females and have an elongated anal fin, the gonopodium, which is used as an intromittent organ during copulation to transfer spermatozeugmata to the female. Gonopodium development is under androgenic stimulation from the testis in the final stages of sexual maturation (Turner, 1941), and can be inhibited by exposure to estrogenic chemicals (Doyle and Lim, 2002). The length of the gonopodium in exposed mosquitofish has therefore been used to measure estrogenicity in water samples.

Early life stage fish are exposed to the samples until they reach maturation, which takes about 80-90 d in mosquitofish (Doyle and Lim, 2002). The length of the gonopodium is then measured and compared with a control group.

A significant shortening of the gonopodium and impairment of terminal hook development has been reported in fish exposed to 100 ng/L E2 (Doyle and Lim 2002). Changes to the morphology of the gonopodium are permanent in the mosquitofish (Doyle and Lim, 2002). Estrogen-induced changes in gonopodium morphology, and particularly the absence of terminal hooks, significantly reduce the reproductive ability of impaired males (Doyle and Lim, 2002).

A.1.4 Sexual Behavior (Doyle and Lim, 2002)

As well as a reduction of gonopodium development, exposure to estrogenic chemicals results in a significant reduction in sexual activity of male *Gambusia* (Doyle and Lim, 2002). Following exposure to E2, sexual activity for each male was assessed by observing the frequency of copulatory attempts (gonopodial thrusting) towards adult females. A significant reduction of copulatory attempts has been shown at concentrations as low as 100ng/L E2 (Doyle and Lim, 2002). This fairly simple endpoint has an immediate impact on population dynamics, as decreased sexual behaviour impacts reproductive fitness (Doyle and Lim, 2002).

A.1.5 Life-cycle Tests (Ankley and Johnson, 2004)

Life cycle tests offer an integrated approach to *in vivo* testing, by exposing the test fish to the samples throughout their life cycle, and thus making sure no window of sensitivity is missed. In life cycle tests, fish are exposed during their entire life cycle, and these tests thus require pre-established conditions and expertise in maintenance and successful breeding of the test species in the laboratory. It is generally convenient to use smaller fish species that have shorter life-spans, such as Japanese medaka (*O.latipes*), fathead minnows (*P. promelas*), sheepshead minnows (*C. variegates*), and zebrafish (*D. rerio*) (Ankley and Johnson, 2004). Life cycle tests are particularly useful as they attempt to bridge the gap between organism-level effects and population-level effects. For example, fecundity (egg production) incorporates effects in both male and female fish, and can affect population-level endpoints such as reproductive success.

Partial life-cycle tests (PLC)

Active reproduction is a critical window of sensitivity to hormonally-active chemicals, and PLCs exploit this window of sensitivity. After a short-term exposure (usually 21 d), several endpoints can be measured, such as fecundity (number of eggs spawned), fertility (number of fertile eggs produced), and hatch (number of fertile eggs to produce larvae). Some of the other endpoints discussed above (such as plasma Vtg or gonad histopathology) can also be measured at the end of the exposure period. Exposure to estrogenic chemicals at this stage usually results in a reduction in fecundity in females (for example fathead minnows exposed to 100 ng/L of E2 have significantly reduced egg production compared to control fish) (Kramer et al., 1998) and an increase in plasma Vtg in males (reviewed in Ankley and Johnson, 2004).

Full life-cycle tests (FLC)

Full life cycle tests provide the most comprehensive approach to detecting the effects of possible EDCs on fish, but are very resource intensive and usually take 6 to 9 months to complete (Ankley and Johnson, 2004). Therefore, although they provide the ultimate assessment of estrogenicity, they may not be suitable to biomonitoring where rapid results are required.

A.1.6 Other Bioassays with Fish

There are several other endpoints that have been shown to be estrogen-responsive. Sexual behaviour for example has been shown to decrease in male mosquitofish (Doyle and Lim, 2002) and Japanese medaka (Oshima et al., 2003) exposed to estradiol. Changes in circulating levels of steroids, sperm counts, or gonad weight (usually expressed as gonadosomatic index, or GSI) have also been used when comparing exposed and reference fish to show exposure-induced hormonal imbalance (McMaster et al., 2001; Toft and Guillette, 2005). However, while these assays undoubtedly point to endocrine disruption, they do not specifically identify estrogenic chemicals.

A.2 Invertebrates

Despite their ecological importance, little is known about the reproductive endocrinology of invertebrates (deFur, 2004). Estrogen-related receptors (ERRs) that have a 2D structure like ERs have been found in invertebrates, however none have been shown to bind steroids, including estrogens.

Several studies in the literature indicate that estradiol may have a regulatory factor in vitellogenin synthesis and reproduction in molluscs. Exposure to estradiol by injection increased vitellogenin synthesis in the Japanese scallop (Matsumoto et al., 1997) and by addition to sea water increased vitellogenin synthesis in the Pacific Oyster (Li et al., 1998). Compounds in the surface waters in an urban area are estrogenic to the freshwater mussel *Elliptio complanata* (Gagné et al., 2001a). Moreover, exposure to relatively low concentrations of estradiol for 72 hours increased the level of alkali labile phosphoprotein (vitellin-like proteins) in *E. complanata* (Gagné et al., 2001b).

Endogenous steroids and estrogen-like receptors have been identified in molluscs, indicating that classical estrogen signalling involved in the reproductive functions is conserved in both vertebrates and invertebrates (Tosti et al., 2001; Di Cosmo et al., 2002). The natural estrogen E2 rapidly affects different immune parameters in molluscan hemocyctes, cells responsible for innate immunity (Canesi et al., 2004a,b), suggesting that estrogens may play a crucial role in endocrine-immune interactions in molluscs.

Sexual development in several freshwater amphipods has been shown to be modified by the environmental estrogen 17α-ethinylestradiol (EE2). Watts et al. (2002) exposed laboratory populations of the freshwater amphipod *Gammarus pulex* to EE2 for 100 days, observing significant increases in the proportion of female amphipods at the higher EE2 concentrations. Sexual development in the freshwater amphipod *Hyalella azteca* was affected by exposure to the synthetic estrogen EE2 in a multigenerational experiment (Vandenbergh et al., 2003). The development of male secondary sex characteristics was repressed and the sex ratio tended to favour female development. In addition to external sex characteristics, the morphology of the reproductive tract in *H. azteca* was affected with indications of hermaphroditism and disturbed maturation of germ cells and spermatogenesis in male amphipods.

Molecular screening tools related to the action of crustacean hormones or their agonists on vitellogenesis, reproduction and development have been developed. Polyclonal antisera against vitellins have been produced for a number of estuarine crustaceans, including ghost shrimp (*Lepidophthalmus louisianensis*), grass shrimp (*Palaemonetes pugio*), crabs (*Rhithropanopeus harrisii* and *Uca panacea*) and mysids (*Americamysis bahia*) (Tuberty et al., 2002). An enzyme-linked immunosorbent assay for lipovitellin quantification has been developed in copepods (Volz and Chandler, 2004). Billinghurst et al. (2000) reported elevated expression of cyrid major protein, a vitellin-like protein, in the larvae of the barnacle *Balanus amphitrite* on exposure to the xeno-estrogen 4-n-nonylphenol (4-NP) at concentrations which significantly reduced their settlement rate (Billinghurst et al., 1998). Similarly, an upregulation of vitellin-like larval storage protein expression was observed in the larvae of the glass prawn, *Palaemon elegans*, exposed to 4-NP (Sanders et al., 2005).

A European research project to identify endocrine disrupting effects in aquatic organisms evaluated estrogenic impacts on (partial) life cycle and multigenerational studies with invertebrates by assessing a range of developmental and reproductive parameters (Segner et al. 2003). Low-dose effects were observed in full life cycle experiments, particularly in the second generation. Results from this project suggested that full life cycle experiments appeared to be the most appropriate exposure regime to reveal sublethal effects of environmental estrogens on invertebrates.

More basic endocrinology research is needed before invertebrate *in vivo* tests can be recommended for screening for estrogenic chemicals.

A.3 Amphibians

Vtg induction (Mitsui et al., 2003)

Male frogs (Mitsui et al., 2003) and newts (Mosconi et al., 2002) also exhibit a significant and dose-dependent increase in plasma Vtg after exposure to estrogenic chemicals. Adult male *Xenopus laevis* are exposed to the sample for 7 d. After exposure, plasma Vtg concentration is measured by ELISA and compared with plasma Vtg in frogs exposed to an E2 standard. Frogs exposed for 7 d to 200-500 ng/L of E2 exhibited a significant induction of plasma Vtg.

Sexual differentiation (Kloas et al., 1999)

Just like fish, amphibians exposed to estrogenic chemicals during the sensitive phase of larval development can exhibit a bias in sex ratio towards females (Kloas et al., 1999; Mosconi et al., 2002). Sex ratio in frogs exposed during larval development can therefore be used to determine estrogenicity. *X. laevis* tadpoles (stage 38/40) are exposed to the samples for approximately 12 weeks, at the end of which sex is determined by examining individuals under a microscope.

Exposure to approximately 30 μg/L of E2 results in almost complete female sex distribution, while exposure to approximately 3 μg/L of E2 results in significant skewing of sex

ratio towards females. Exposure to industrial estrogen mimics (such as nonylphenol, octylphenol, and bisphenol A) also resulted in a significant female bias (Kloas et al., 1999). This assay provides a simple assay for estrogenicity in amphibians using a widely available test species (*X. laevis*).

A.4 Other Vertebrates

In vivo bioassays based on higher vertebrates may not be ethically and financially appropriate for testing the large numbers of samples that would be expected in a routine environmental monitoring program (Korach and McLachlan, 1995). Therefore, other tests discussed previously (such as *in vitro* mammalian cell bioassays and *in vivo* tests with lower vertebrates) are more ethically suitable for use as a screen, while these more complex *in vivo* bioassays would be extremely useful for weight-of-evidence data to support initial *in vitro* assays.

Reptiles

Although estrogenic chemicals clearly also have effects on reptiles, as shown by complete sex reversal in *Caiman latrostris* exposed to estradiol (Stoker et al., 2003), reptile-based bioassays may not be an appropriate lab models for use in routine water monitoring due to difficulties in procuring aquatic reptiles to a laboratory environment.

Birds

Even though some bird species are clearly associated with the water environment for some or all of their life cycle, it is unclear at the moment how relevant a bird-based bioassay would have in an environmental water-monitoring scheme. Birds synthesize Vtg, and the female genital tract has been weighed for years as a bioassay of estrogenicity, so both these endpoints could potentially be used to measure estrogenicity in water.

Mammals

The rodent uterotrophic assay is one of the best *in vivo* assays for demonstrating estrogenic activity (Korach and McLachlan, 1995; Owens and Koeter, 2003). There are substantial historical data available to support the relevance and reliability of the assay, it can address metabolism and feedback questions, and has undergone an extensive multi-laboratory validation process through a cooperative project between the OECD and U.S. EPA (Owens and Koeter, 2003). Along with other mammalian-based bioassays, these form the core of the EDSTAC-recommended battery of bioassays to test individual chemicals for endocrine disrupting activity (NIEHS, 2002).

A.5 References for Appendix II

Alberti M, Kausch U, Haindl S, and Seifert M. 2005. Gene expression analysis for exposure to estrogenic substances. Acta Hydrochimica et Hydrobiologica 33: 38-44.

Andersen L, Holbech H, Gessbo A, Norrgren L, and Petersen GI. 2003. Effects of exposure to 17 alpha-ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology Part C 134: 365-374.

Angus RA, Weaver SA, Grizzle JM, and Watson RD. 2002. Reproductive characteristics of male mosquitofish (*Gambusia affinis*) inhabiting a small southeastern US river receiving treated domestic sewage effluent. Environmental Toxicology and Chemistry 21: 1404-1409.

Ankley GT, and Johnson RD. 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. Ilar Journal 45: 469-483.

Arukwe A, Knudsen FR, and Goksøyr A. 1997. Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. Environmental Health Perspectives 105: 418-422.

Ataria J, Gooneratne R, and Tremblay LA. 2004. Development of a specific ELISA for shortfin eel (*Anguilla australis*) vitellogenin. Australasian Journal of Ecotoxicology 10: 43-52.

Barnhoorn IEJ, Bornman MS, Pieterse GM, and van Vuren JHJ. 2004. Histological evidence of intersex in feral sharptooth catfish (Clarias gariepinus) from an estrogen-polluted water source in Gauteng, South Africa. Environmental Toxicology 19: 603-608.

Baroiller JF, and D'Cotta H. 2001. Environment and sex determination in farmed fish. Comparative Biochemistry and Physiology Part C 130: 399-409.

Billinghurst Z, Clare AS, Fileman T, McEvoy J, Readman J, and Depledge MH. 1998. Inhibition of barnacle settlement by the environmental oestrogen 4-nonylphenol and the natural oestrogen 17beta-oestradiol. Marine Pollution Bulletin 36: 833-839.

Billinghurst Z, Clare AS, Matsumura K, and Depledge MH. 2000. Induction of cypris major protein in barnacle larvae by exposure to 4-n-nonylphenol and 17beta-oestradiol. Aquatic Toxicology 47: 203-212.

Bond CE. 1996. *Biology of fishes*. Saunders College Publishing, Philadelphia, PA, USA.

Bortone SA, and Davis WP. 1994. Fish intersexuality as indicator of environmental stress. Bioscience 44: 165-172.

Bowman CJ, and Denslow ND. 1999. Development and validation of a species- and gene-specific molecular biomarker: Vitellogenin mRNA in largemouth bass (*Micropterus salmoides*). Ecotoxicology 8: 399-416.

Brooks BW, Foran CM, Peterson BN, Weston J, La Point TW, and Huggett DB. 2003. Linkages between population demographics and municipal effluent estrogenicity. Bulletin of Environmental Contamination and Toxicology 71: 504-511.

Canesi L, Ciacci C, Betti M, Cecilia Lorusso L, Marchi B, Burattini S, Falcieri E, and Gallo G. 2004a. Rapid effects of 17beta-estradiol on cell signaling and function of Mytilus hemocyctes. General and Comparative Endocrinology 136: 58-71.

Canesi L, Cecilia Lorusso L, Ciacci C, Betti M, Zampini M, and Gallo G. 2004b. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. General and Comparative Endocrinology 138: 58-69.

deFur PL. 2004. Use and role of invertebrate models in endocrine disruptor research and testing. Ilar Journal 45: 484-493.

Denslow ND, Bowman CJ, Ferguson RJ, Lee HS, Hemmer MJ, and Folmar LC. 2001a. Induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) treated with 17beta-estradiol, diethylstilbestrol, or ethinylestradiol: The use of mRNA fingerprints as an indicator of gene regulation. General and Comparative Endocrinology 121: 250-260.

Denslow ND, Chow MJ, Kroll KJ, and Green L. 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8: 385-398.

Denslow ND, Lee HS, Bowman CJ, Hemmer MJ, and Folmar LC. 2001b. Multiple responses in gene expression in fish treated with estrogen. Comparative Biochemistry and Physiology Part B 129: 277-282.

Denton TE, Howell WM, Allison JJ, McCollum J, and Marks B. 1985. Masculinization of female mosquitofish by exposure to plant sterols and *Mycobacterium smegmatis*. Bulletin of Environmental Contamination and Toxicology 35: 627-632.

Di Cosmo A, Di Cristo C, and Paolucci M. 2002. A estradiol-17beta receptor in the reproductive system of the female of Octopus vulgaris: characterization and immunolocalization. Molecular Reproduction and Development 61: 367-375.

Doyle CJ, and Lim RP. 2002. The effect of 17 beta-estradiol on the gonopodial development and sexual activity of *Gambusia holbrooki*. Environmental Toxicology and Chemistry 21: 2719-2724.

Drèze V, Monod G, Cravedi JP, Biagianti-Risbourg S, and Le Gac F. 2000. Effects of 4-nonylphenol on sex differentiation and puberty in mosquitofish (*Gambusia holbrooki*). Ecotoxicology 9: 93-103.

Flouriot G, Pakdel F, Ducouret B, Ledrean Y, and Valotaire Y. 1997. Differential regulation of two genes implicated in fish reproduction: Vitellogenin and estrogen receptor genes. Molecular Reproduction and Development 48: 317-323.

Folmar L, Denslow N, Rao V, Chow M, Crain D, Enblom J, Marcino J, and Guillette LJ. 1996. Vitellogenin induction and reduced serum testosterone concentration in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environmental Health Perspectives 104: 1096-1101.

Folmar LC, Denslow ND, Kroll K, Orlando EF, Enblom J, Marcino J, Metcalfe C, and Guillette LJJ. 2001. Altered serum sex steroids and vitellogenin induction in walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant. Archives of Environmental Contamination and Toxicology 40: 392-398.

Folmar L, Hemmer M, Hemmer R, Bowman C, Kroll K, and Denslow N. 2000. Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an *in vivo*, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. Aquatic Toxicology.

Gagné F, Marcogliese DJ, Blaise C, and Gendron AD. 2001a. Occurence of compounds estrogenic to freshwater mussels in surface waters in an urban area. Environmental Toxicology 16: 260-268.

Gagné F, Blaise C, Lachance B, Sunahara GI, and Sabik H. 2001b. Evidence of coprostanol estrogenicity to the freshwater mussel Elliptio complanata. Environmental Pollution 115: 97-106.

Hadley ME. 1988. *Endocrinology*. Prentice Hall, Englewood Cliffs, NJ, USA.

Hansen PD, Dizer H, Hock B, Marx A, Sherry J, McMaster M, and Blaise C. 1998. Vitellogenin - a biomarker for endocrine disruptors. Trends in Analytical Chemistry 17: 448-451.

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge RJ, Rycroft R, Sumpter JP, and Tylor T. 1996. A survey of estrogenic activity in United Kingdom inland waters. Environmental Toxicology and Chemistry 15: 1993-2002.

Hartley WR, Thiyagarajah A, Anderson MB, Broxson MW, Major SE, and Zell SI. 1998. Gonadal development in Japanese medaka (*Oryzias latipes*) exposed to 17 beta-estradiol. Marine Environmental Research 46: 145-148.

Hemming JM, Allen HJ, Thuesen KA, Turner PK, Waller WT, Lazorchak JM, Lattier D, Chow M, Denslow N, and Venables B. 2004. Temporal and spatial variability in the estrogenicity of a municipal wastewater effluent. Ecotoxicology and Environmental Safety 57: 303-310.

Heppell SA, Denslow ND, Folmar LC, and Sullivan CV. 1995. Universal assay of vitellogenin as a biomarker for environmental estrogens. Environmental Health Perspectives 103: 9-15.

Higashitani T, Tamamoto H, Takahashi A, and Tanaka H. 2003. Study of estrogenic effects on carp (*Cyprinus carpio*) exposed to sewage treatment plant effluents. Water Science and Technology 47: 93-100.

Holbech H, Andersen L, Petersen GI, Korsgaard B, Pedersen KL, and Bjerregaard P. 2001. Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology Part C 130: 119-131.

Hsiao CD, and Tsai HJ. 2003. Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism in vivo. Developmental Biology 262: 313-323.

Hurter E, Pool EJ, and Van Wyk JH. 2002. Validation of an ex vivo Xenopus liver slice bioassay for environmental estrogens and estrogen mimics. Ecotoxicology and Environmental Safety 53: 178-187.

Islinger M, Yuan H, Voelkl A, and Braunbeck T. 2002. Measurement of vitellogenin gene expression by RT-PCR as a tool to identify endocrine disruption in Japanese medaka (*Oryzias latipes*). Biomarkers 7: 80-93.

Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brighty G, Sumpter J, and Tyler C. 2002. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. Biology of Reproduction 66: 272-281.

Jobling S, Nolan M, Tyler C, Brighty G, and Sumpter J. 1998. Widespread sexual disruption in wild fish. Environmental Science and Technology 32: 2498-2506.

Kime D. 1999. A strategy for assessing the effects of xenobiotics on fish reproduction. Science of the Total Environment 225: 3-11.

Kime D, Nash J, and Scott A. 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. Aquaculture 177: 345-352.

Kloas W, Lutz I, and Einspanier R. 1999. Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. Science of the Total Environment 225: 59-68.

Korach KS, and McLachlan JA. 1995. Techniques for detection of estrogenicity. Environmental Health Perspectives 103(S7): 5-8.

Korsgaard B, and Pedersen K. 1998. Vitellogenin in *Zoarces viviparus*: Purification, quantification by ELISA and induction by estradiol-17 beta and 4-nonylphenol. Comparative Biochemistry and Physiology Part C 120: 159-166.

Kramer VJ, Miles-Richardson S, Pierens SL, and Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (Pimephales promelas) exposed to waterborne 17beta-estradiol. Aquatic Toxicology 40: 335-360.

Larkin P, Folmar L, Hemmer M, Poston A, Lee H, and Denslow N. 2002. Array technology as a tool to monitor exposure of fish to xenoestrogens. Marine Environmental Research 54: 395-399.

Lattier D, Gordon D, Burks D, and Toth G. 2001. Vitellogenin gene transcription: A relative quantitative exposure indicator of environmental estrogens. Environmental Toxicology and Chemistry 20: 1979-1985.

Legler J, Broekhof JLM, Brouwer A, Lanser PH, Murk AJ, Van der Saag PT, Vethaak AD, Wester P, Zivkovic D, and Van der Burg B. 2000. A novel in vivo bioassay for (xeno-)estrogens using transgenic zebrafish. Environmental Science & Technology 34: 4439-4444.

Leusch FDL, Chapman HF, Kay GW, Gooneratne SR, and Tremblay LA. 2006. Anal fin morphology and gonadal histopathology in mosquitofish (Gambusia holbrooki) exposed to treated municipal sewage effluent. Archives of Environmental Contamination and Toxicology. doi:10.1007/s00244-005-1040-5.

Leusch FDL, van den Heuvel MR, Laurie AD, Chapman HF, Gooneratne SR, and Tremblay LA. 2005. Quantification of vitellogenin mRNA induction in mosquitofish (Gambusia affinis) by reverse transcription real-time polymerase chain reaction (RT-PCR). Biomarkers 10(6): 429-438.

Li Q, Osada M, Suzuki T, and Mori K. 1998. Changes in vitellin during oogenesis and effect of estradiol-17beta on vitellogenesis in the Pacific oyster, Crassostrea gigas. Invertebrate Reproduction and Development 33: 87-93.

Lomax D, Roubal W, Moore J, and Johnson L. 1998. An enzyme-linked immunosorbent assay (ELISA) for measuring vitellogenin in English sole (*Pleuronectes vetulus*): development, validation and cross-reactivity with other pleuronectids. Comparative Biochemistry and Physiology Part B 121: 425-436.

MacKay M, Raelson J, and Lazier C. 1996. Up-regulation of estrogen receptor mRNA and estrogen receptor activity by estradiol in liver of rainbow trout and other teleostean fish. Comparative Biochemistry and Physiology Part C 115: 201-209.

Matsumoto T, Osada M, Osawa Y, and Mori K. 1997. Gonadal estrogen profile and immunohistochemical localization of steriodogenic enzymes in the oyster and scallop during sexual maturation. Comparative Biochemistry and Physiology B 118: 811-817.

McClain J, Oris J, Burton G, and Lattier D. 2003. Laboratory and field validation of multiple molecular biomarkers of contaminant exposure in rainbow trout (*Oncorhynchus mykiss*). Environmental Toxicology and Chemistry 22: 361-370.

McMaster M, Jardine J, Ankley G, Benson W, Greeley M, Gross T, Guillette L, MacLatchy D, Orlando E, Van Der Kraak G, and Munkittrick K. 2001. An interlaboratory study on the use of steroid hormones in examining endocrine disruption. Environmental Toxicology and Chemistry 20: 2081-2087.

Metcalfe C, Metcalfe T, Kiparissis Y, Koenig B, Khan C, Hughes R, Croley T, March R, and Potter T. 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with Japanese medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 20: 297-308.

Mitsui N, Tooi O, and Kawahara A. 2003. Sandwich ELISAs for quantification of Xenopus laevis vitellogenin and albumin and their application to measurement of estradiol-17 beta effects on whole animals and primary-cultured hepatocytes. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 135: 305-313.

Mosconi G, Carnevali O, Franzoni M, Cottone E, Lutz I, Kloas W, Yamamoto K, Kikuyama S, and Polzonetti-Magni A. 2002. Environmental estrogens and reproductive biology in amphibians. General and Comparative Endocrinology 126: 125-129.

Nakari T. 2004. Estrogenicity of municipal effluents assessed *in vivo* and *in vitro*. Environmental Toxicology 19: 207-215.

NIEHS. 2002. Current status of test methods for detecting endocrine disruptors. Expert panel evaluation of the validation status of in vitro test methods for detecting endocrine disruptors. National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA.

Nilsen BM, Berg K, Eidem JK, Kristiansen SI, Brion F, Porcher JM, and Goksøyr A. 2004. Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. Analytical and Bioanalytical Chemistry 378: 621-633.

Nolan M, Jobling S, Brighty G, Sumpter J, and Tyler C. 2001. A histological description of intersexuality in the roach. Journal of Fish Biology 58: 160-176.

Oshima Y, Kang I, Kobayashi M, Nakayama K, Imada N, and Honjo T. 2003. Suppression of sexual behavior in male Japanese medaka (*Oryzias latipes*) exposed to 17 beta-estradiol. Chemosphere 50: 429-436.

Owens W, and Koeter HBWM. 2003. The OECD program to validate the rat uterotrophic bioassay: An overview. Environmental Health Perspectives 111(12): 1527-1529.

Pait A, and Nelson J. 2003. Vitellogenesis in male *Fundulus heteroclitus* (killifish) induced by selected estrogenic compounds. Aquatic Toxicology 64: 331-342.

Panter G, Thompson R, and Sumpter J. 2000. Intermittent exposure of fish to estradiol. Environmental Science and Technology 34: 2756-2760.

Petrovic M, Solé M, de Alda M, and Barceló D. 2002. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: Integration of chemical analysis and biological effects on feral carp. Environmental Toxicology and Chemistry 21: 2146-2156.

Piferrer F. 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197: 229-281.

Porter C, and Janz D. 2003. Treated municipal sewage discharge affects multiple levels of biological organization in fish. Ecotoxicology and Environmental Safety 54: 199-206.

Purdom C, Hardiman P, Bye V, Eno N, Tyler C, and Sumpter J. 1994. Estrogenic effects of effluents from sewage treatment works. Chemistry and Ecology 8: 275-285.

Robinson C, Brown E, Craft J, Davies I, Moffat C, Pirie D, Robertson F, Stagg R, and Struthers S. 2003. Effects of sewage effluent and ethynyl oestradiol upon molecular markers of oestrogenic exposure, maturation and reproductive success in the sand goby (*Pomatoschistus minutus*, Pallas). Aquatic Toxicology 62: 119-134.

Rodgers-Gray T, Jobling S, Morris S, Kelly C, Kirby S, Janbakhsh A, Harries J, Waldock M, Sumpter J, and Tyler C. 2000. Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. Environmental Science and Technology 34: 1521-1528.

Rodgers-Gray T, Jobling S, Kelly C, Morris S, Brighty G, Waldock M, Sumpter J, and Tyler C. 2001. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environmental Science and Technology 35: 462-470.

Routledge E, Sheahan D, Desbrow C, Brighty G, Waldock M, and Sumpter J. 1998. Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. Environmental Science and Technology 32: 1559-1565.

Sanders MB, Billinghurst Z, Depledge MH, and Clare AS. 2005. Larval development and vitellin-like protein expression in Palaemon elegans larvae following xeno-estrogen exposure. Integrative and Comparative Biology 45: 51-60.

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

Segner H, Caroll K, Fenske M, Janssen CR, Maack G, Pascoe D, Schafers C, Vandenbergh GF, Watts M, and Wenzel A. 2003. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicology and Environmental Safety 54: 302-314.

Sherry J, Gamble A, Fielden M, Hodson P, Burnison B, and Solomon K. 1999. An ELISA for brown trout (*Salmo trutta*) vitellogenin and its use in bioassays for environmental estrogens. Science of the Total Environment 225: 13-31.

Shilling A, and Williams D. 2000. Determining relative estrogenicity by quantifying vitellogenin induction in rainbow trout liver slices. Toxicology and Applied Pharmacology 164: 330-335.

Solé M, Porte C, and Barceló D. 2001. Analysis of the estrogenic activity of sewage treatment works and receiving waters using vitellogenin induction in fish as a biomarker. Trends in Analytical Chemistry 20: 518-525.

Stoker C, Rey F, Rodriguez H, Ramos JG, Sirosky P, Larriera A, Luque EH, and Munoz-de-Toro M. 2003. Sex reversal effects on Caiman latirostris exposed to environmentally relevant doses of the xenoestrogen bisphenol A. General and Comparative Endocrinology 133: 287-296.

Thomas-Jones E, Walkley N, Morris C, Kille P, Cryer J, Weeks I, and Woodhead J. 2003. Quantitative measurement of fathead minnow vitellogenin mRNA using hybridization protection assays. Environmental Toxicology and Chemistry 22: 992-995.

Thorpe K, Cummings R, Hutchinson T, Scholze M, Brighty G, Sumpter J, and Tyler C. 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environmental Science and Technology 37: 1142-1149.

Tilton F, Benson W, and Schlenk D. 2002. Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. Aquatic Toxicology 61: 211-224.

Todorov J, Elskus A, Schlenk D, Ferguson P, Brownawell B, and McElroy A. 2002. Estrogenic responses of larval sunshine bass (*Morone saxatilis* x *M-Chrysops*) exposed to New York city sewage effluent. Marine Environmental Research 54: 691-695.

Toft G, and Guillette LJ. 2005. Decreased sperm count and sexual behavior in mosquitofish exposed to water from a pesticide-contaminated lake. Ecotoxicology and Environmental Safety 60: 15-20.

Tosti E, Di Cosmo A, Cuomo A, Di Cristo C, and Gragnaniello G. 2001. Progesterone induces activation in Octopus vulgaris spermatozoa. Molecular Reproduction and Development 59: 97-105.

Tremblay L, and Van Der Kraak G. 1998. Use of a series of homologous *in vitro* and *in vivo* assays to evaluate the endocrine modulating actions of beta-sitosterol in rainbow trout. Aquatic Toxicology 43: 149-162.

Tuberty SR, Nates SF, and McKenney, CL Jr. 2002. Polyclonal antisera against estuarine crustacean vitellins: a molecular approach to reproductive endocrinology and toxicology. In: Modern Approaches to the Study of Crustacea. E Escobar and F Alvarez (Eds.), Kluwer Academic/Plenum Publishers, pp 29-37.

Turner C. 1941. Morphogenesis of the gonopodium in *Gambusia affinis affinis*. Journal of Morphology 69: 161-185.

Tyler CR, and Sumpter JP. 1990. The development of a radioimmunoassay for carp, Cyprinus carpio, vitellogenin. Fish Physiology and Biochemistry 8: 129-140.

Ueno T, Yasumasu S, Hayashi S, and Iuchi I. 2004. Identification of choriogenin cis-regulatory elements and production of estrogen-inducible, liver-specific transgenic Medaka. Mechanisms of Development 121: 803-815.

van Aerle R, Nolan M, Jobling S, Christiansen L, Sumpter J, and Tyler C. 2001. Sexual disruption in a second species of wild cyprinid fish (the Gudgeon, *Gobio gobio*) in United Kingdom freshwaters. Environmental Toxicology and Chemistry 20: 2841-2847.

van Aerle R, Pounds N, Hutchinson T, Maddix S, and Tyler C. 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. Ecotoxicology 11: 423-434.

Van den Belt K, Verheyen R, and Witters H. 2001. Reproductive effects of ethynylestradiol and 4t-octylphenol on the zebrafish (*Danio rerio*). Archives of Environmental Contamination and Toxicology 41: 458-467.

Van den Belt K, Wester P, van der Ven L, Verheyen R, and Witters H. 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): Time dependency and reversibility. Environmental Toxicology and Chemistry 21: 767-775.

Vandenbergh GF, Adriaens D, Verslycke T, and Janssen CR. 2003. Effects of 17alpha-ethinylestradiol on sexual development of the amphipod Hyalella azteca. Ecotoxicology and Environmental Safety 54: 216-222.

Verslycke T, Vandenbergh G, Versonnen B, Arijs K, and Janssen C. 2002. Induction of vitellogenesis in 17 alpha-ethinylestradiol-exposed rainbow trout (*Oncorhynchus mykiss*): a method comparison. Comparative Biochemistry and Physiology Part C 132: 483-492.

Vethaak A, Rijs G, Schrap S, Ruiter H, Gerritsen A, and Lahr J. 2002. Estrogens and xeno-estrogens in the aquatic environment of The Netherlands: Occurence, potency and biological effects. RIZA/RIKZ, Lelystad, The Netherlands.

Vethaak AD, Lahr J, Schrap SM, Belfroid AC, Rijs GBJ, Gerritsen A, de Boer J, Bulder AS, Grinwis GCM, Kuiper RV, Legler J, Murk TAJ, Peijnenburg W, Verhaar HJM, and de Voogt P. 2005. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 59: 511-524.

Volz DC, and Chandler GT. 2004. An enzyme-linked immunosorbent assay for lipovitellin quantification in copepods: A screening tool for endocrine toxicity. Environmental Toxicology and Chemistry 23: 298-305.

Watts MW, Pascoe D, and Carroll K. 2002. Population responses of the freshwater amphipod Gammarus pulex (L.) to an environmental oestrogen, 17alpha-ethinylestradiol. Environmental Toxicology and Chemistry 21: 445-450.

Yadetie F, Arukwe A, Goksøyr A, and Male R. 1999. Induction of hepatic estrogen receptor in juvenile Atlantic salmon *in vivo* by the environmental estrogen, 4-nonylphenol. Science of the Total Environment 233: 201-210.

Table 2. Summary of Main Characteristics of Selected *in vivo* Bioassays.

Name	Page	Reference	Estimated DL [1]	Use	Duration	Pros	Cons	Env. Ref. [2]
Vtg induction assay	54	Multiple references including Hansen et al. 1998; Kime et al. 1999; Solé et al. 2001	Varies with species, but usually 10 – 100 ng/L	+++	Varies, but usually 4 – 21 d	- Endpoint easily measured - High biological relevance - Incorporates metabolism and transport - Widely used	- Work intensive - Requires aquaculture laboratory	Multiple references including Brooks et al. 2003; Folmar et al. 1996; Hansen et al. 1998; Harries et al. 1996; Hemming et al. 2004; Higashitani et al. 2003; McClain et al. 2003; Nakari 2004; Purdom et al. 1994; Rodgers-Gray et al. 2000; Routledge et al. 1998; Tilton et al. 2002; Todorov et al. 2002
Zrp induction assay	57	Arukwe et al. 1997	Unknown, but appears to be very sensitive (see text)	+	Varies, usually 7 – 21 d	- High biological relevance - Incorporates metabolism and transport	- Work intensive - Requires aquaculture laboratory	
ER induction assay	57	MacKay et al. 1996	Unknown, but appears slightly less sensitive than Zrp or Vtg (see text)	-	Varies, usually 7 – 21 d	- High biological relevance - Incorporates metabolism and transport	- Work intensive - Requires aquaculture laboratory	Todorov et al. 2002
Transgenic luminescent zebrafish	57	Legler et al. 2000	300 ng/L	+	2-4 d	- Good biological relevance - Allows detailed analysis of estrogen-responsive organs - Incorporates metabolism and transport	- Work intensive - Requires aquaculture laboratory - Requires PC2 facilities - Requires high level of expertise	Vethaak et al. 2002

Name	Page	Reference	Estimated DL [1]	Use	Duration	Pros	Cons	Env. Ref.
Transgenic medaka	58	Ueno et al. 2004	10,000 ng/L	-	2-3 d	- Good biological relevance - Incorporates metabolism and transport	- Work intensive - Requires aquaculture laboratory - Requires PC2 facilities - Requires high level of expertise	
Gonadal sex ratio and intersex	58	Multiple references including Andersen et al. 2003; Bortone and Davis 1994; Hartley et al. 1998; Jobling et al. 1998; Metcalfe et al. 2001; Nolan et al. 2001; van Aerle et al. 2002	Varies with species, but can be as low as 10 ng/L	+++	> 60 d	- Good ecological relevance	- Very long term exposure - Work intensive - Requires aquaculture laboratory	Multiple references, including Barnhoorn et al. 2004; Jobling et al. 2002; Jobling et al. 1998; Rodgers-Gray et al. 2001
Secondary sexual characteristics in poeciliids	59	Doyle and Lim 2002	100 ng/L	++	80 – 90 d	- Fair ecological relevance	- Very long term exposure - Work intensive - Requires aquaculture laboratory	Angus et al. 2002; Leusch et al. 2006
Sexual behaviour	60	Doyle and Lim 2002	100 ng/L	+	80 – 90 d	- Good ecological relevance	- Very long term exposure - Work intensive - Requires aquaculture laboratory - Specificity of the measured response?	Toft and Guillette 2005

Name	Page	Reference	Estimated DL [1]	Use	Duration	Pros	Cons	Env. Ref.
Vtg induction in frogs	62	Mitsui et al. 2003	200 – 500 ng/L	-	7 d	- High biological relevance	- Requires maintenance of frog population - Requires aquaculture laboratory - Work intensive	
Sexual differentiation in frogs	62	Kloas et al. 1999	2,000 ng/L	-	7 – 10 d	- Good ecological relevance	- Requires maintenance of frog population - Requires aquaculture laboratory - Work intensive	

[1] Estimated detection limit with 17b-estradiol for that test duration.
[2] Relevant environmental references where that type of assay has been used to determine estrogenicity of environmental water samples.

Global Water Research Coalition
c/o International Water Association
Alliance House
12 Caxton Street
London SW1H 0QS
United Kingdom
tel: +44 207 654 5545
email: gwrc@iwahq.org.uk
www.globalwaterresearchcoalition.net