**Hazardous Substances Series**

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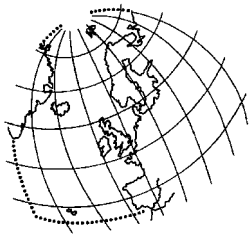
# Survey of the use of effect related methods to assess and monitor wastewater discharges

*Testing of endocrine effects*



OSPAR Commission
2003

The Convention for the Protection of the Marine Environment of the North-East Atlantic (the "OSPAR Convention") was opened for signature at the Ministerial Meeting of the former Oslo and Paris Commissions in Paris on 22 September 1992. The Convention entered into force on 25 March 1998. It has been ratified by Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, Netherlands, Norway, Portugal, Sweden, Switzerland and the United Kingdom and approved by the European Community and Spain.

*La Convention pour la protection du milieu marin de l'Atlantique du Nord-Est, dite Convention OSPAR, a été ouverte à la signature à la réunion ministérielle des anciennes Commissions d'Oslo et de Paris, à Paris le 22 septembre 1992. La Convention est entrée en vigueur le 25 mars 1998. La Convention a été ratifiée par l'Allemagne, la Belgique, le Danemark, la Finlande, la France, l'Irlande, l'Islande, le Luxembourg, la Norvège, les Pays-Bas, le Portugal, le Royaume-Uni de Grande Bretagne et d'Irlande du Nord, la Suède et la Suisse et approuvée par la Communauté européenne et l'Espagne.*



ISBN 1-904426-23-9

# contents

# 1. DEFINITIONS AND ABBREVIATIONS

Endocrine-disrupting chemicals are defined as (1) exogenous substances that cause adverse health effects in an intact organism or its progeny, consequent to changes in endocrine functions (EU, 1997) or as (2) exogenous agents that interfere with the production, release, transport, metabolism, binding and action or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and regulation of developmental processes (EPA, 1996a).

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| AR: | Androgen Receptor |
| ACTH: | Adrenocorticotrophin |
| ASTM: | American Society for Testing and Materials |
| CHH: | Crustacean Hyperglycemic Hormone |
| DIN: | Deutsches Institut für Normung (German Institute for Standardisation) |
| DRP: | Detailed Review Paper |
| EDC: | Endocrine-Disrupting Chemical |
| EDTA: | Task Force on Endocrine Disruptor Testing and Assessment |
| ELISA: | Enzyme-linked Immunosorbent Assay |
| ER: | Estrogen Receptor |
| ERA: | Environmental Risk Assessment |
| EPA: | Environmental Protection Agency, USA |
| EU: | European Union |
| FETAX: | Frog Embryo Teratogenesis Assay |
| GH: | Growth Hormone |
| GSI: | Gonadosomatic Index |
| GtH: | Gonadotrophin |
| HSI: | Hepatosomatic Index |
| IEG: | Intersessional Expert Group |
| JH: | Juvenile Hormone |
| MF: | Methyl Farnesoate |
| MOA: | Mode of Action |
| mRNA: | Messenger Ribonucleic Acid |
| NTP: | National Toxicology Program (USA) |
| OECD: | Organisation for Economic Co-operation and Development |
| OSPAR: | Oslo and Paris Convention for the Protection of the of Marine Environment of the North-East Atlantic |
| PBT: | Persistence, Liability to Bioaccumulate, Toxicity |
| PDS: | Point and Diffuse Sources |
| PPP: | Plant Protection Products |
| RIA: | Radioimmunoassay |
| SETAC: | Society of Environmental Toxicology and Chemistry |
| $T_3$: | Triiodothyronine |
| $T_4$: | Thyroxine |
| TBT: | Tributyltin |
| TSH: | Thyroid Stimulating Hormone |
| VTG: | Vitellogenin |
| WEA: | Whole Effluent Assessment |
| WHO: | World Health Organisation |
| WWTP: | Waste Water Treatment Plant |

## 2. EXECUTIVE SUMMARY

The objective of this background document was to prepare a survey on endocrine test methods for the evaluation of waste water within whole effluent assessment. The survey focused on fish and aquatic invertebrates as potential test organisms for the assessment whether a chemical or an effluent has endocrine-disruptive potential and whether the observed effects are related to ecologically more relevant endpoints used in environmental risk assessment schemes.

Most data were derived from activities initiated by national research programmes of the United States of America, various countries within the European Union and the Organisation for Economic Co-operation and Development. These research activities resulted in numerous scientific papers and workshop reports on the evaluation of endocrine effects on aquatic organisms caused by individual chemicals or by effluents from waste water treatment plants.

The present survey revealed that for the time being standardised and validated test methods designed specifically to identify endocrine effects in aquatic organisms are not available. However, in the scientific literature many *in vitro* and *in vivo* methods have been described, which show the potential of becoming eventually a tool to be used in whole effluent assessment. A way forward would be the development and the standardisation of a set of *in vitro* and *in vivo* screening methods which allow the identification of endocrine disrupting chemicals by disclosing their specific mode of action in relation to the various endocrine systems occurring in aquatic vertebrate and invertebrate animals. Based on data derived from these screening assays, secondary tests (*in vivo* tests) are recommended to be performed in a next step. Secondary tests would provide results which can be used in environmental risk assessments and which could also be applicable to whole effluent assessment. When comparing OSPAR's compilation of test methods used by various countries for whole effluent assessment with the methods assessing endocrine effects in the scientific literature, three test methods were identified which have the potential, and after modifications could become tests, to address specifically endocrine effects induced by waste water.

## 2. RÉCAPITULATIF

Le projet de document de fond avait pour objectif de préparer une étude des méthodes de tests endocriniens pour l'évaluation des eaux usées dans le contexte de l'évaluation des effluents entiers. L'étude a été centrée sur le poisson et les invertébrés aquatiques comme organismes tests potentiels afin de savoir si un produit chimique ou un effluent est capable de perturber le système endocrinien et si les effets observés sont liés à des points finals écologiquement plus pertinents, appliqués dans les régimes d'évaluation des risques environnementaux. Pour la plupart, les données ont été tirées d'activités lancées soit dans le cadre des programmes nationaux de recherche des Etats-Unis d'Amérique et de divers pays de l'Union européenne, soit du programme de l'Organisation de Coopération et de Développement Economiques. Ces travaux ont abouti à de nombreuses communications scientifiques et comptes rendus d'ateliers, dans lesquels les effets endocriniens sur des organismes aquatiques, provoqués par tel ou tel produit chimique ou par des effluents provenant de stations d'épuration des eaux usées, ont été évalués.

L'étude a démontré qu'il n'existait pour l'instant pas de méthodes de test normalisées et validées, conçues spécifiquement pour déceler les effets endocriniens chez les organismes aquatiques. Néanmoins, de nombreuses méthodes *in vitro* et *in vivo* ont été décrites dans la bibliographie scientifique, méthodes qui pourraient en définitive devenir des outils exploitables pour l'évaluation des effluents entiers. Il a été proposé de créer et/ou de normaliser et de valider une série de méthodes de dépistage *in vitro* et *in vivo* permettant de déterminer les produits chimiques perturbateurs endocriniens en mettant en évidence leur mode d'action spécifique sur les divers systèmes endocriniens, tels qu'ils se produisent chez des animaux aquatiques vertébrés et invertébrés. Compte tenu des données tirées des analyses de dépistage, il est recommandé, comme étape suivante, de procéder à des tests secondaires (tests *in vivo*). Les tests secondaires permettraient d'obtenir des résultats pouvant être exploités pour les évaluations des risques environnementaux, et pouvant aussi être applicables à l'évaluation des effluent entiers. Lorsque l'on a comparé les méthodes de test collationnées par OSPAR et appliquées dans divers pays pour l'évaluation des effluents entiers, aux méthodes d'évaluation des effets endocriniens décrites dans la bibliographie scientifique, l'on a trouvé trois méthodes de test qui ont le potentiel recherché, et qui, après modification, pourraient devenir des tests visant spécifiquement les effets endocriniens provoqués par les eaux usées.

# 3. PREAMBLE AND THE OBJECTIVE OF THE BACKGROUND DOCUMENT

At the meeting of the working group on Point and Diffuse Sources (PDS) in Cambridge, U.K., on 11-15 December 2000, the progress report from the Intersessional Expert Group (IEG) on Whole Effluent Assessment (WEA) was presented (PDS 00/12/3-E). In this report, among other things, further work concerning a survey on applied methods and the development of methods on tests for genotoxicity and estrogenic activity was proposed. Germany volunteered to carry the work forward and to prepare a background document on a survey on endocrine test methods including methods for testing estrogenic activities for the evaluation of wastewater within WEA.

## 3.1 Sources of data and information

Important review papers for the preparation of this background document were written by ANKLEY et al. (1998) and DEFUR et al. (1999). Valuable information was further contained in two OECD reports on 'Expert Consultation on Endocrine Disruptors Testing in Fish' (OECD 1999 and 2000). For 1999, 2000 and the first six months of the year 2001 a literature search was conducted using Current Contents for Windows (Institute for Scientific Information, Inc., Philadelphia, Pennsylvania, USA), Section Agriculture, Biology & Environmental Sciences. The background document quotes papers cited by ANKLEY et al. (1998) and DEFUR et al. (1999) which are thought to be valuable for an in-depth understanding of the subject, but which have not been assessed in detail by the author of this document. All information provided in the background document has been published in peer-reviewed scientific journals or textbooks except the reports provided by OECD.

# 4. BACKGROUND INFORMATION

## 4.1 Introduction

### *4.1.1 Endocrine disruption in aquatic wildlife*

Most examples of endocrine disruption in wildlife have come from animals living in or closely associated with the aquatic environment (ANKLEY and GIESY, 1998). This can be explained by the fact that freshwater and marine environments are repositories for large volume discharges of many chemicals which, depending on the nature of the chemical, can be incorporated in aquatic organisms via the diet and via the water across the skin and gill surfaces (MCKIM and ERICKSON, 1991). Furthermore, the eggs of most aquatic (and some semi-aquatic) animals are deposited into the water and the developing embryos may thus be directly exposed to various chemicals at vulnerable stages in their development.

Examples for incidences of endocrine disruption that have been observed in aquatic ecosystems at the individual and population levels are: (1) wild male fish with measurable levels of vitellogenin, a female-specific protein, in different riverine environments receiving municipal effluents in the United Kingdom and the United States (PURDOM et al., 1994; FOLMAR et al., 1996); (2) female mosquito fish (*Heterandria formosa*) in streams dominated by pulp mill effluents, which possessed male-specific gonadopodia (BORTONE et al., 1989; BORTONE and DAVIS, 1994); (3) white sucker (*Catostomus commersoni*) exposed to pulp mill effluents, which exhibited delayed sexual maturation, reduced gonadal growth, and altered steroidogenic capacity (MCMASTER et al., 1996; VAN DER KRAAK et al., 1998); (4) the alligator population of the Lake Apopka, Florida, USA, exposed to a spill of organochlorine pesticides, which showed morphological abnormalities and decreased reproduction (GUILLETTE et al., 1994); (5) imposex (development of male genitalia by females) and decreased reproductive success observed in some marine gastropod species exposed to the organotin TBT (BRYAN et al., 1986; FENT, 1996).

The most clearly defined example of endocrine disruption in European wildlife is the case of imposex in marine prosobranch molluscs, where combined field and laboratory studies have proven that sexual disruption in these animals has resulted from exposure to TBT (OEHLMANN et al., 1996). Furthermore, this exposure has resulted in the extinction of mollusc populations in some of the more heavily polluted marine environments (BRYAN et al., 1986; MINCHIN et al., 1996). In areas where TBT use was banned and the resulting contamination reduced, a restoration of some mollusc populations has followed (MINCHIN et al., 1995; EVANS et al., 2000).

### *4.1.2 Identification and characterisation of endocrine disrupting chemicals*

In an attempt to establish a priority list of substances for further evaluation of their role in endocrine disruption, the European Commission (EU, 2000a) composed a working list of 564 substances that showed potential and suspected endocrine disruption. Based on the criteria 'highly persistent' and/or 'high production volume' 146 substances were selected from the working list. For 66 of these 146 substances at least one study provided evidence of endocrine disruption in an intact organism. From *in vitro* studies, it was concluded that further 51 of the 146 substances had the potential for endocrine disruption in intact organisms. Taking into consideration physico-chemical properties, bioaccumulation potential and degradation in the environment as well as use, production volume, emissions and monitoring data, 29 substances and substance groups of the 66 substances exhibiting evidence of endocrine disruption in an intact organism were evaluated as being of high concern for exposure. Twenty three substances with high concern of exposure have been recorded in the aquatic environment (EU, 2000a).

### *4.1.3 The endocrine system of vertebrates (in particular fish)*

The primary function of the endocrine system is to transform various exogenous stimuli (visual, tactile, pheromonal, and nutritive cues) into chemical messages, the hormones. This includes a chain of events, which result in the expression of the appropriate gene or in the activation of already existing tissue specific enzyme systems. The endocrine system represents an important tool for the timely coordination of development (e.g. induction of spawning cycles or sexual maturity) and metabolism (e.g. glucose homeostasis).

The hierarchical structure of components of the endocrine system, which are involved in fish reproduction is shown schematically in Figure 1. On the molecular level, the endocrinology of teleost fish is similar to that of mammals, but it diverges significantly at higher levels of biological organisation (REINBOTH, 1980; SPINDLER, 1997; KIME, 1998). As in other oviparous vertebrates, the reproductive cycles of the male and female teleosts are complex and highly coordinated, and rely upon the integrated activities of the pituitary, controlled by the hypothalamus and higher brain centres, and the gonads (REDDING and PATINO, 1993; VAN DER KRAAK et al., 1998). Several developmental strategies exist among fishes. These include oviparity, viviparity, and ovoviparity. Some fish are naturally hermaphroditic, others are plastic, reversing sex in response to environmental parameters such as temperature, populational sex ratio or age (TAKAHASHI, 1977; SHAPIRO, 1994).

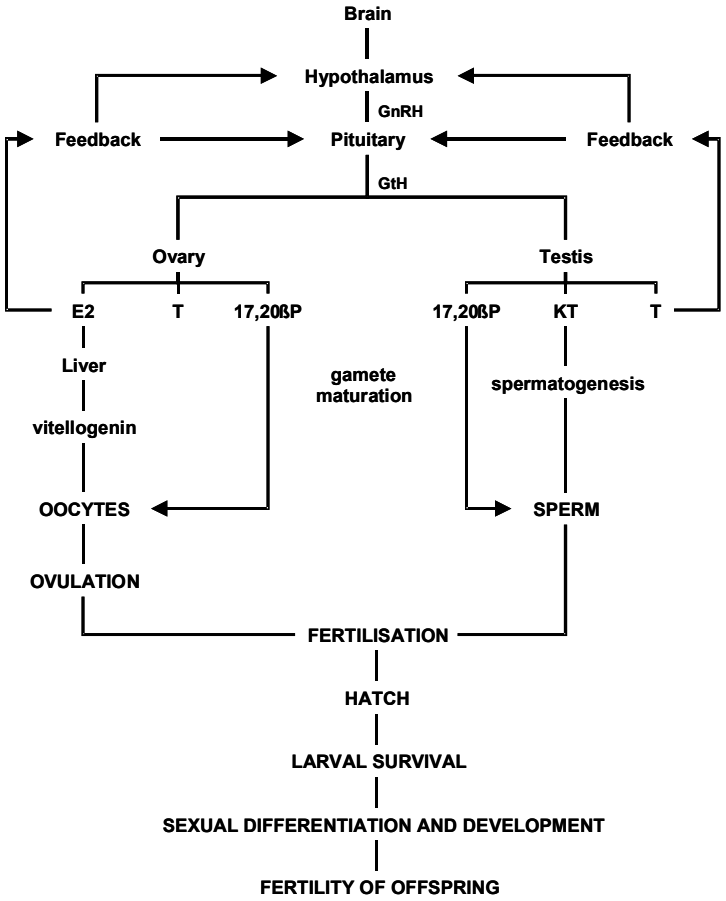


**Figure 1**: The reproductive system of fish and possible sites of action of endocrine-active compounds according to KIME (1998). Abbreviations: **GnRH**: gonadotrophin-releasing hormone; **GtH**: gonadotrophin; **E2**: estradiol; **T**: testosterone; **KT**: 11-ketotestosterone; **17,20βP**: 17,20βP-dihydroxy-4-pregnen-3-one.

Although gonadotrophin (GtH) and the gonadal steroids (e.g. estradiol, testosterone) play the major role in reproductive development, their actions may be modulated by a range of other endocrine factors (Figure 2). Stress, for example, has an inhibitory effect on reproduction (CAMPBELL et al., 1992 and 1994a), probably acting via adrenocorticotrophin (ACTH) or cortisol secreted by the interrenal gland or, in fish, by the anterior kidney. Furthermore, the thyroid hormones thyroxine ($T_4$) and triiodothyronine ($T_3$) affect reproduction and larval development, while the growth hormone (GH) affects the development of fish and its offspring. Consequently some of the reported effects of pollution on reproduction could be caused by stress and/or by effects on the thyroid gland and/or by effects related to the GH possibly mediated by the pituitary and the hypothalamus (KIME, 1998).

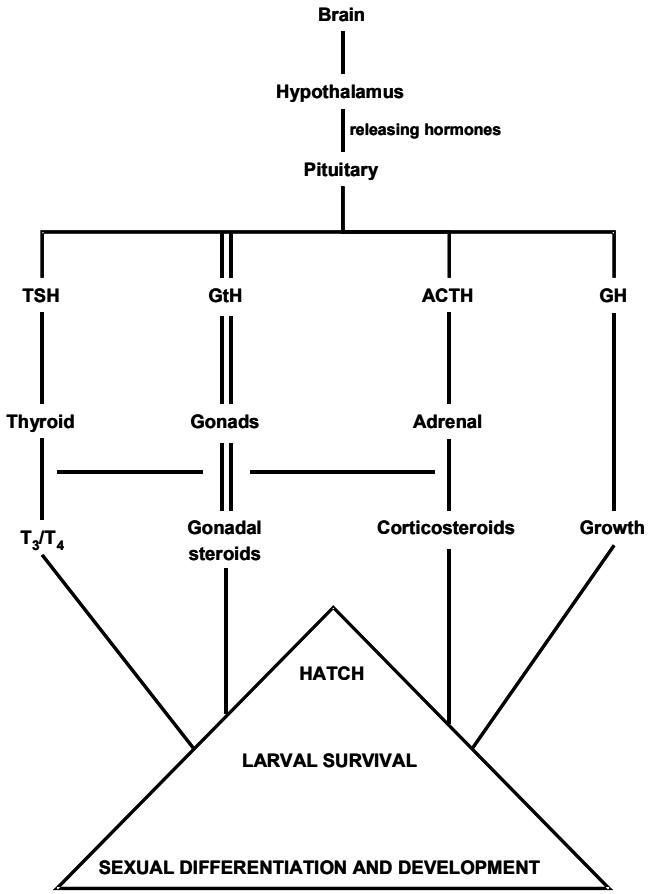


**Figure 2**: Further endocrine effects on fish reproduction according to KIME (1998). Abbreviations: **TSH**: thyroid stimulating hormone; **GtH**: gonadotrophic hormone; **ACTH**: adrenocorticotrophin; **GH**: growth hormone; **$T_3$/$T_4$**: triiodothyronine/thyroxine.

### *4.1.4 The endocrine system of invertebrates*

Invertebrates constitute more than 95% of animal life on earth and, therefore, it is not surprising that regulation of endocrine processes in invertebrates is considerably more diverse than in vertebrates (see REINBOTH, 1980; GORBMAN and DAVEY, 1991). The knowledge of invertebrate endocrinology has been driven by the need to control invertebrate populations, particularly insect pests. Many insecticides interfere with some aspect of insect endocrinology, resulting ultimately in disruption of development and/or reproduction. There is also a significant amount of information available on the endocrinology of some crustaceans, particularly the decapods, of which many species are commercially and recreationally important. However, information is usually limited to a few species with little known about interspecies variability in endocrinology. Information on the endocrinology of other invertebrate groups is often limited and fragmentary (LEBLANC et al., 1999).

Invertebrates have developed a multitude of unique endocrine-controlled approaches to growth, development and reproduction including processes of metamorphosis, diapause, limb regeneration and pupation. Other endocrine-regulated processes, such as carbohydrate metabolism, gonadal development, and vitellogenin synthesis, seem to be common among many of the invertebrates as well as relevant vertebrate taxa. The neuroendocrine system in invertebrates is more diverse than that found in the vertebrates (HIGHNAM and HILL, 1977; LAUFER and DOWNER, 1989). Many aquatic and terrestrial invertebrates have complicated life histories, display various forms of hermaphrodism (GORBMAN and DAVEY, 1991), and in some cases, sexual dimorphism (SELLMER, 1967; ECKELBARGER, 1974). Their reproductive cycles can be highly complex (SASTRY, 1968 and 1970) and controlled by various environmental stimuli, including light intensity, temperature, desiccation, and diet (ANSELL and TREVALLION, 1967; LARGEN, 1967; COPELAND and BECHTEL, 1974; YOUNG, 1978; TESSIER et al., 1983).

Invertebrates use terpenoids, steroid hormones and peptide hormones, of which by far the most common invertebrate hormones are peptides which are secreted by neurosecretory structures, cells and tissues of neurological origin. A wide variety of neuropeptides function as endocrine regulators in all invertebrate groups that have been studies so far. These neurohormones include molt-stimulating or inhibiting -hormones (arthropods), allotropins and allostatins (insects and other invertebrates), diuretic and antidiuretic hormones (insects and others), cardioacceleratory peptides (molluscs, insects, and others), insulin-like peptides (mollusks), egg-laying-stimulating hormones (mollusks), regeneration-stimulating hormones (annelids and crustaceans), and glycine-leucine tryptophan amides or metamorphosis-stimulating hormones (coelenterates). Neuropeptides may directly regulate an endocrine process or modulate the production or secretion of the terminal hormone (e.g., ecdysone or juvenile hormone) (LEBLANC et al., 1999).

Vertebrate-type hormones have been detected in a number of invertebrate taxa (VOOGT et al., 1985; DE LOOF and DE CLERCK, 1986; HINES et al., 1992); however, firm evidence of their role in the endocrine system of most invertebrates is lacking (PINDER and POTTINGER, 1998). Androgenic and estrogenic hormones have been found in every invertebrate class examined thus far (LEBLANC, 1998). In various species androgens control male secondary sex characteristics, and in echinoderms and gastropods, estrogens increase oocyte growth (LEBLANC, 1998). Unique hormones in invertebrates are, for example, ecdysones and related compounds termed "ecdysteroids" found in arthropods, nematodes and molluscs, which mediate differentiation, growth, reproduction, vitellogenesis and molting (CHANG and O'CONNOR, 1988; CHARNIAUX-COTTON and PAYEN, 1988). All invertebrates that periodically shed an exoskeleton use ecdysteroids to regulate the molting process. In addition, those species that use ecdysteroids also produce terpenoids including juvenile hormone (JH), farnesyl acetone, farnesonic acid and methyl farnesoate (MF), which are used to regulate embryogenesis, development and reproduction (WIGGLESWORTH, 1970; BOWERS, 1990).

### *4.1.5 EDCs in EU and OSPAR documents related to environmental policy*

Endocrine effects have been included in the criteria to define possible hazardous substances by Annex VIII of the Water Framework Directive (EU, 2000b) and by OSPAR's "Safety Net Procedure for the Inclusion of Substances in the List of Substances of Possible Concern" (OSPAR, 2002) which is related to "OSPAR Strategy with regard to Hazardous Substances" (OSPAR, 1998). In both cases EDCs can be listed as priority substance (e.g. EU, 2001) even if the so called PBT selection criteria are not fulfilled.

### *4.1.6 Driving forces for the development of tests to measure effects of EDCs*

In 1996, OECD established the Task Force on Endocrine Disrupters Testing and Assessment (EDTA) with, *inter alia*, the objective of developing new and revised existing test guidelines to detect endocrine disrupters. The work on test guidelines is predominantly carried out by two recently formed groups, which report to the EDTA: a) The Validation Management Group for Screening and Testing of

Endocrine Disrupters for Mammalian Effects (VMG-mam); b) The Validation Management Group for the Screening and Testing of Endocrine Disrupters for Ecotoxicological Effects (VMG-eco). OECD focuses on *in vivo* test methods and does not work on the validation and standardisation of any *in vitro* test method. Details and progress of OECD's work on EDCs can be found when visiting the internet (http://webnet1.oecd.org/EN/document/0,,EN-document-524-14-no-24-6687-0,00.html).

In 1996, US Congress included specific language on endocrine disruption in the Food Quality Protection Act and amended Safe Drinking Water Act. The former mandated that US EPA developed an endocrine disruptor screening program, whereas the latter authorised US EPA to screen endocrine disruptors found in drinking water sources. US EPA formed an advisory committee, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to develop recommendations for a screening program, which were finalised in August 1998. EDSTAC's recommendations were used to design the US EPA Endocrine Disruptor Screening Program of which elements have been implemented. For example, draft detailed review papers on several *in vivo* tests (frog metamorphosis assay, fish screening assays; life cycle toxicity test with shrimps) and *in vitro* estrogen and androgen receptor binding assays has been published in recent years. All papers and the progress of US EPA's work on EDCs can be obtained from the internet (http://www.epa.gov/scipoly/oscpendo/).

### *4.1.7 Existing test methods and endocrine disruption*

Based on the definition for EDCs (EPA, 1996; EU, 1997) endocrine effects are characterised by interference(s) of chemicals with the functions of the natural hormones, which, consequently, cause adverse health effects in an intact organism or its progeny. As a result, the traditional ecotoxicological endpoints (e.g. mortality) studied in short-term toxicity tests (e.g. EU, 1992; OECD, 1992a) performed at the lower tiers of the ERA schemes for chemicals (EU, 1993a, b), PPPs (EU, 1991), veterinary products (EU, 1998a) and biocides (EU, 1998b) are not sufficient to detect endocrine effects of chemical substances. Measurement endpoints like growth and reproduction determined at higher tier studies can be related to endocrine effects. However, higher tier studies are either conducted for high production volume chemicals or to refute risks determined at lower tiers of the ERA procedure. Hence, endocrine effects of chemicals can easily be overlooked, when ERA based on traditional short-term toxicity tests indicate no toxicity and higher tier studies are not required.

Three activities ought to be highlighted, which have been aimed at developing specific test methods for identifying potential endocrine-disrupting chemicals and to measure endocrine effects in intact organisms. The first activity was initiated by the U.S. Congress in 1996 (see Section 4.1.6). Major results of this activity are summarised in a report to the U.S. Congress (EPA, 2000). The second activity was initiated by the 25th Joint Meeting of the OECD Chemicals Group and Management Committee in November 1996, which resulted in the Detailed Review Paper (DRP) (OECD, 2001). The main focus of the recommendations given by the DRP is on modifying existing sub-chronic and chronic OECD Test Guidelines for detecting the effects of endocrine disrupting substances on the hormonal control of reproduction. Finally, the third activity was initiated by SETAC. It resulted in the Proceedings from the International SETAC Workshop on Endocrine Disruption in Invertebrates (DEFUR et al., 1999).

In the following, (proposed) testing methods will be described, of which the majority is based on the above mentioned activities and which have been published in the scientific literature. Testing methods related to mammalians, birds, and reptiles are not taken into consideration, because tests with these organisms have not been applied to assess the toxicity of waste water discharges. Additionally, modifications of these tests, which would include specific endpoints related to the endocrine system, are far from being standardised.

## 4.2 Screening methods to identify EDCs

An ideal screening test should be relatively rapid and cost-effective so that many chemicals can be tested. A screening design should not consist of a single endpoint, but rather of a suite of endpoints reflective of the mechanisms of concern (e.g., [anti-] estrogenicity/androgenicity, thyroid activity). The endpoints should be relatively easy to measure to facilitate development of standardised screening protocols and inter-laboratory validation. Finally, screening tools should be appropriate for hazard identification. Positive results in a screen with a particular chemical would result in secondary testing aimed at better characterisation of specific effects. In this context, screening tests should be biased in favour of minimising the occurrence of false negatives (ANKLEY et al., 1998).

An important consideration in the identification of a suite of screening tests is the degree to which extrapolation of (anti-) estrogenic/androgenic and other endocrine effects among species is possible. Many aspects of endocrine function are conserved among species (NORRIS, 1996; VAN DER KRAAK et al., 1998) and this similarity serves as a potentially useful basis for among-species extrapolation (KAVLOCK and ANKLEY, 1996; ANKLEY et al., 1997). Nevertheless, there are differences among taxa in terms of endocrine function, mainly downstream from the hormone receptor at the levels of physiological and cellular responses (REINBOTH, 1980; KIME, 1987). There may also considerable interspecies specific differences in sensitivity towards endocrine-active chemicals.

### *4.2.1 In vitro testing methods*

#### *4.2.1.1 Receptor binding affinity*

Possible (anti-) estrogens/androgens can be identified by measuring the relative binding affinity to the estrogen receptor (ER) or androgen receptor (AR) using competitive ligand binding techniques. Increasing concentrations of the chemical in question are included in incubation mixtures consisting of radiolabelled ligand (e.g. 17ß estradiol or dihydroxytestosterone) and a cytosolic or nuclear preparation from a tissue containing ER or AR. The free radiolabelled ligand is separated from the bound ligand and expressed as the percentage displaced by the competing chemical. The ligand binding assays have widespread acceptance. ER and AR from mammalian and non-mammalian species including fish, reptiles, and birds are used (EROSCHENKO and PALMITER, 1980; THOMAS and SMITH, 1993; VONIER et al., 1996). The assays do not distinguish between agonists and antagonists and do not assess whether metabolites of test chemicals which may occur under physiological conditions show endocrine potential. Establishing a standard operating procedure for a competitive ligand binding assay would be a difficult task given species- and situation-dependent diversity in tissue expression levels of receptors. For example, in fish ER- and AR-levels in various tissues are known to vary widely throughout a seasonal cycle (LAZIER et al., 1985; POTTINGER, 1988; RILEY and CALLARD, 1988; SMITH and THOMAS, 1991) and ER and AR binding affinities can also vary seasonally in a variety of species (YU and HO, 1989; CAMPBELL et al., 1994b). In the future, the use of recombinant receptors from different species may eliminate some of these confounding factors, as well as provide a supply of receptors without animal sacrifice (ZACHAREWSKI, 1997).

Recently summaries on *in vitro* estrogen and androgen receptor binding assays have been provided by NTP (2002a, b).

#### *4.2.1.2 Cellular proliferation*

These assays measure proliferation in cell lines that are dependent upon hormones for stimulation of growth. The most well-known test, the E-Screen, is based on the human breast cancer cell line MCF-7, which requires estrogens to proliferate (SOTO et al., 1992). Hence, estrogenic xenobiotics cause the cells to proliferate, whereas antiestrogens inhibit the proliferation response to 17ß-estradiol. Although this

assay is sensitive and can distinguish between ER-agonists and -antagonists, there are disadvantages due to the sensitivity of the assay to culture conditions (ZACHAREWSKI, 1997). In addition, cell lines responsive to other hormones (e.g. androgens) are currently not available. Because of these limitations, cell proliferation assays are, at present, not acceptable as screens for wildlife.

#### *4.2.1.3 Gene expression*

Gene expression assays measure the induction of gene transcription following hormone receptor activation (ZACHAREWSKI, 1997). One technique used to assess gene expression is measurement of mRNA of an endogenous gene product stimulated by estrogens or androgens. Reporter gene assays in eukaryotic cell lines or yeast also can be used to assess gene expression. In the reporter gene assay, the response element does not induce the transcription of the endogenous product, but instead mRNA for an enzyme (the reporter). This mRNA is translated into the respective enzyme, such as luciferase, chloramphenicol acetyltransferase, ß-galactosidase, or alkaline phosphatase. When an appropriate substrate is added, the enzyme catalyses a light-emitting, radioactive, or colorimetric reaction that indicates the amount of gene expression. Reporter gene assays have been used by investigators to detect estrogenic activity of single chemicals and complex mixtures (ZACHAREWSKI et al., 1995; ROUTLEDGE and SUMPTER, 1996; GRAY et al., 1997).

Reporter gene assays with yeast cells (YES-assays) were used to determine the estrogenic potential of sewage sludge as well as the influent and effluent of WWTPs (REHMANN, et al,. 1999a; REHMANN, et al,. 1999b; THOMAS et al., 2001; WITTERS ET AL., 2001; MURK et al., 2002).

Gene expression assays in eukaryotic cell lines have the advantage over receptor binding assays of being able to distinguish between agonists and antagonists. Because of widespread use in the pharmaceutical industry for drug screening, protocols are relatively standardised and easily adapted to automated microtiter plate formats allowing large numbers of samples to be processed quickly. Disadvantages of gene expression assays include the specialised equipment and training required for both mRNA detection and reporter gene endpoints. Moreover, most of the reporter gene assays are based on transient transfections for inserting plasmids; this is a labour-intensive procedure and introduces interassay variation into the results. Although the lack of background levels of endogenous hormones and receptors in yeast limits some confounding variables, results between yeast and mammalian cells are sometimes poorly correlated, which may in part be due to differences in transport of xenobiotics across cell walls and cell membranes, and to differences in receptor populations (ZACHAREWSKI, 1997). Little work has been carried out with these types of assays in non-mammalian species, thus limiting their utility as screening tools in wildlife.

Recently, summaries on *in vitro* estrogen and androgen receptor transcriptional activation assays have been provided by NTP (2002c, d).

#### *4.2.1.4 Steroidogenic stimulation/inhibition*

To detect interference of xenobiotics with the oxidative synthetic enzymes of the steroidogenic pathway the use of *ex vivo* assays has been recommended. For these assays laboratory animals are exposed *in vivo* and excised gonads would then be incubated *in vitro* to assess relevant enzyme activities. Following addition of radiolabelled precursors to the incubation media, intermediates or final products in pathways of concern would be detected using methods such as thin-layer chromatography or radioimmunoassay. The advantages of these assays include their reproducibility and simplicity. They also enable investigation of both female (ovary) and male (testes) function. This type of *ex vivo* and *in vitro* steroidogenesis assay has been applied for example by (MCMASTER et al., 1996) who collected fish near pulp and paper mills. The disadvantage of this assay is related to the fact that the *in vivo* regulation of intact organs may differ from that in excised tissues. Metabolic activities observed *in vitro* might thus not be relevant under *in vivo* conditions.

#### *4.2.1.5 Advantages and disadvantages of in vitro screening methods*

*In vitro* models are test systems for studying the actual mechanisms of endocrine-modulating activity. Interactions of interest can be studied independently of the many possible elements that can interfere with the identification of the actual modes of action (MOAs). In addition, *in vitro* models can facilitate obtaining reproducible empirical data in a time- and cost-effective manner. In fact, it should be noted that DIN has started a procedure to standardise *in vitro* assays related to determine endocrine effects. However, *in vitro* models have limitations as predictive tools in risk assessment: they fail to account for the complexity of the whole animal and several important mechanisms inherent to *in vivo* systems. Cellular and cell-free systems lack the intact signalling pathway with regard to intercellular interactions and endocrine homeostasis. Moreover, most *in vitro* models lack the ability to metabolically alter chemicals. As a result, extrapolation from *in vitro* to *in vivo* systems can lead to false negatives for compounds that are bioactivated, and it can overestimate the potency of compounds which are readily degraded *in vivo*. For screening-level assessments at the molecular level the uncertainty in extrapolations across species may be acceptable, because mechanisms involved in the synthesis, release, and action of hormones are similar in target cells of most vertebrate wildlife. Because of differences in endocrine systems among species, and metabolic and toxicokinetic variations extrapolation of effects at the cellular, tissue, and whole organism level is more problematic. Therefore, *in vitro* tests cannot be the sole basis of a primary screening scheme. Thus, *in vitro* tests could serve as a component of a tiered screening effort consisting of short-term *in vivo* as well as *in vitro* assays. Furthermore, the screening scheme for wildlife should include *in vivo* assays with one or more model species representative of the various animal classes of concern. Indications of endocrine activity in both *in vitro* and short-term *in vivo* assays would be sufficient to identify a chemical for further testing. In principal, these considerations which are focused on single chemicals are also relevant for mixtures of chemicals which occur in effluents of sewage treatment plants.

### *4.2.2 In vivo testing methods*

The objective of *in vivo* screening assays is the identification of potential endocrine disrupting activity as a signal, which triggers the need to conduct higher tier testing. It is not the purpose of screening to provide data on adverse effects of endocrine functions for environmental risk assessment. This is the role of higher tier testing. A screening assay should be a sensitive and cost-effective short-term test.

#### *4.2.2.1 Fish tests*

The OECD requires *in vivo* tests with teleost fish for various regulatory programs, including short-term lethality, early life-stage tests, as well as partial and full-life-cycle studies (OECD, 1992a, b, c). Commonly tested species include carp (*Cyprinus carpio*), guppy (*Poecilia reticulata*), rainbow trout (*Oncorhynchus mykiss*), sheepshead minnow (*Cyprinodon variegatus*), fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*). Endpoints used in standard partial and full life-cycle tests include mortality, behaviour, growth, development and reproduction. Although several of these endpoints could reflect (anti-) estrogenic/androgenic and other endocrine effects, most are relatively non-specific in terms of MOA. Moreover, these long-term tests are too resource-intensive to be considered as screening assays. The purpose of EDC screening tests in fish and other wildlife is to identify potential EDCs and their MOA, but not necessarily prediction of subsequent effects at the population or community level. A draft Detailed Review Paper on fish screening assay for endocrine disruption has been published be US EPA (2002a).

Based on the DRP (OECD, 2001) the Task Force on Endocrine Disruptor Testing and Assessment (EDTA), which was established under the auspices of the OECD Test Guidelines Programme, organised two meetings of experts on testing in fish (OECD, 1999 and 2000a). The task of these meetings was to propose *in vivo* fish tests for both the screening and the secondary testing tier to identify potential EDCs and to assess endocrine effects in fish, respectively.

For the development of fish *in vivo* screening assays, OECD experts recommended the drafting of two protocols for juvenile fish (based on OECD, 1984 and 2000) and adult fish. The recommended species for the juvenile assay are carp, fathead minnow, medaka, rainbow trout and zebrafish. For the adult assay the recommended species are fathead minnow, medaka and zebrafish. All-female or all-male strains are particularly useful to study effects of endocrine disruptors on sex differentiation.

Based on results derived from acute or chronic tests the fish screening assays should include the testing of three concentrations below the solubility limit of the test chemical. The exposure route should be environmentally relevant, preferably in ambient water, although intraperitoneal injection and dietary exposure is acceptable for hydrophobic substances. It is desirable to test positive and negative control substances to verify that the assay is working. For example, ethinylestradiol tested in the range of 5-20 ng/l could be a positive control substance for estrogen/anti-estrogen effects, whereas dihydroxytestosterone could be a reference substance for androgen effects.

In each fish *in vivo* screening assay apical endpoints (e.g. sexual behaviour, sexual characteristics) and specific/mechanistic endpoints (MOAs) should be measured. In addition, it might be useful to distinguish between core and supplementary endpoints. The core endpoints should include gross morphology (e.g. GSI and HSI), VTG measurement and gonad histology, whereas optional supplementary endpoints might include steroid titres, fertility, and others. In the selection of the fish test species, it should be kept in mind that the measurements of the GSI and HSI are species- and size-dependent with different levels of variability. Secondary sexual characters are only observable in sexually dimorphic species, and may also vary depending on the laboratory conditions and the age of the fish. Changes in secondary sexual characters are best observed in mature individuals and in juveniles of known genetic sex.

Gross morphology
Depending on the fish species it was recommended that gross morphology data should include GSI, macroscopic examination of gonads, secondary sexual characteristics (in adults of sexually dimorphic fish species or in juveniles where the genetic sex is known) and HSI (OECD, 2000b). Endpoints related to secondary sex characteristics and gonadal status (GSI, histopathology) are meaningless and/or impossible to measure in juvenile fish. Sexual dimorphism in fish can be affected by exposure to estrogenic or androgenic substances (TURNER, 1942; YAMAMOTO, 1975; PAPOULIAS et al., 1995; MILES-RICHARDSON et al., 1996). In fathead minnows, size of breeding tubercles and fatpads on males diminishes with exposure to 17ß-estradiol (MILES-RICHARDSON et al., 1996). The ratio of gonad weight to body weight (GSI) is applicable to both sexes of oviparous fish species. The GSI is not necessarily specific for any particular MOA and should only be compared in fish that are at the same stage of gametogenesis.

Vitellogenin (VTG) measurement
Induction of vitellogenin has been observed in a variety of fish species (both male and female) exposed via different routes to a number of putative ER agonists (DONOHOE and CURTIS, 1996; JOBLING et al., 1996; NIMROD and BENSON, 1998; HANSEN et al., 1998; SHERRY et al., 1999). The preferred methods for VTG detection involve analyses of blood samples with antibodies to VTG using routine procedures as RIA, ELISA or western blotting. VTG induction has also been quantified by measuring vitellogenin mRNA in rainbow trout (LECH et al., 1996). A less sensitive method for detecting VTG is the measurement of changes in alkaline-labile protein-bound phosphorus (CRAIK and HARVEY, 1984; MOMMSEN and WALSH, 1988). One restriction of VTG induction assays may be caused by the limited amount of blood available from small fish species. However, it may be possible to monitor liver cytosolic fractions (NIMROD and BENSON, 1998). VTG can also be determined in whole body homogenates (TYLER et al., 1999; ANDERSEN et al., 2000; PANTER et al., 2002). Another limitation may occur since VTG structure differs among species and antibodies developed for one fish may or may not cross-react with another, thus requiring species-specific antibody development (SUMPTER, 1985; DENSLOW et al., 1996; TYLER et al., 1996). There is a need for more research to clarify the range of endogenous VTG levels in adult and juvenile fish.

Histology data on gonads
Baseline data on the effects of estrogens on gonad histology exist for zebrafish, fathead minnow and medaka (MILES-RICHARDSON et al. 1999, 2000, SCHOLZ and GUTZEIT 2000, ISLINGER et al. 2001, LÄNGE et al. 2001, JOON KANG et al. 2002, VAN DER VEEN and WESTER 2002, WESTER et al. 2002). VAN DEN BELT et al. (2002) demonstrated the histological effects of the strongest known ER agonist 17α-ethinylestradiol on the testes and ovary of zebrafish: following 3 days of exposure to 8,7 ng/L of ethinylestradiol VTG levels were increased both in male and female fish and effects on ovary histology were recorded, while effects on testes histology were only seen after 24 days of exposure. It should be noted that the threshold level for induction of VTG in the zebrafish is in the range of 1,1–3,6 ng/L (FENSKE et al. 2001, DUIS et al. 2002, ROSE et al. 2002), thus below the concentration studied by VAN DEN BELT et al. (2002).

The following optional supplementary endpoints can be applied in fish screening tests.

Plasma steroid concentrations
Alterations in plasma sex steroid concentrations can result from several different MOAs, including direct effects on steroidogenic enzymes or indirect modifications of complex regulatory feedback loops. Plasma steroid concentrations in fish are usually measured using RIA or ELISA with monoclonal antibodies to mammalian 17ß-estradiol and testosterone. In most teleosts, 11-keto-testosterone may be a more appropriate measure of androgenic activity than testosterone (IDLER et al., 1961; ARAI, 1967; IDLER et al., 1976). As with VTG, steroids are more difficult to measure in small species than in large species because of the limited blood volume. Another concern is that fluctuations of steroid concentrations can occur during the course of the reproductive cycle and diurnal rhythms (ZOHAR et al., 1988; MATSUYAMA et al., 1990).

Alteration in sexual differentiation
The hormonal environment of early life stages of fish affects their gonadal and phenotypic development (GRAY and METCALFE, 1996). Hence, sexual differentiation can serve as a potential indicator of ER or AR agonists, although other factors, e.g. water temperature, can also affect sexual development in some fish species (STRUSSMAN and PATINO, 1995). Thus, if such a measurement endpoint was routine, the model species would have to be well characterised with respect to the regulation of sexual differentiation.

Steroidogenesis
Gonadal steroidogenesis in fish can be assessed with both *in vitro* and ex vivo protocols. For example, gonads of wild fish collected from rivers receiving effluents from pulp and paper mills were shown to have diminished steroid-producing capacity (VAN DER KRAAK et al., 1992; MCMASTER et al., 1996). Similar experiments were also performed with fish following laboratory exposure to chemicals (MACLATCHY and VAN DER KRAAK, 1995).

Hypothalamic-pituitary function
A variety of chemicals have been shown to disrupt reproductive endocrine function in teleosts by altering gonadotropin secretion (THOMAS, 1989; VAN DER KRAAK et al., 1992). However, gonadotropin RIAs have been developed for relatively few species. Therefore, the measurement of gonadotropin secretion in routine screening assays does not seem feasible. As research is progressing, it might be possible to add endpoints related to other types of endocrine activity, e.g. androgenic/anti-androgenic and thyroid activity.

#### *4.2.2.2 Amphibian tests*

The standardised and validated frog embryo teratogenesis assay (FETAX) (BANTLE et al., 1994a, b; BANTLE, 1996) is a 96-h whole-embryo developmental toxicity screen that consists of exposure of *Xenopus laevis* eggs with subsequent examination of embryonic development to assess the teratogenic potential of chemicals (ASTM, 1991; BANTLE, 1993). Specific test endpoints are survival, growth and malformations of embryos. *X. laevis* is a good model, because it is commercially available and easy to maintain. In addition, throughout the whole year eggs are plentiful and transparent and an extensive database exists on various aspects of its biology, including molecular aspects of development.

FETAX assay could be modified to include exposure scenarios and endpoints appropriate for the detection of (anti-) estrogens/androgens and other EDCs. Exposure protocols could include injection, feeding or waterborne exposure of both males and females prior to mating. Endpoints in parents could include breeding behaviour, fertilisation rate, GSI, number or weight of released gametes and stage of eggs in ovaries. In addition, more resource-intensive tests could examine parameters of sperm motility, velocity and abnormality. In the offspring, sex ratios could be measured and vitellogenin production assessed. Antibodies are available that are specific for *X. laevis* vitellogenin (PALMER and PALMER, 1995).

To determine the MOAs of EDCs in the amphibian screening assay specific endpoints can be included into the frog embryo teratogenesis assay.

Receptor binding
The ERs and ARs in *X. laevis* have been cloned (WEILER et al., 1987; SAVOURET et al., 1991; PEREZ et al., 1996), and the same binding assays used in other vertebrates could be performed for amphibians. LUTZ and KLOAS (1999) established an estrogen receptor binding assay by using the liver cytosol fraction of *X. laevis* . The method has been applied to identify EDCs and to screen sewage effluents for endocrine disrupting pollutants.

Germinal vesicle breakdown
The hormonal events controlling the onset of meiosis have been well studied in *X. laevis* (GEBAUER and RICHTER, 1997; SAGATA, 1997). Xenobiotics that affect the breakdown of the oocyte nucleus (germinal vesicle) can be assayed either *in vivo* or *in vitro*. These tests would be of short duration, and the results would be interpretable in the context of a large body of literature in this model system.

Vitellogenin (VTG) measurement
As described for fish, male amphibia exhibit increased VTG levels following exposure to estrogenic compounds. In most tests performed so far, the animals were injected on consecutive days with test chemicals of concern and a positive control, blood samples were collected, and vitellogenin is measured with immunodetection techniques such as ELISA, RIA or western blotting. However, little information exists at present concerning cross-reactivity of existing amphibian antibodies for vitellogenin. Induction of vitellogenin in *X. laevis* has also been assessed by detection of VTG mRNA (RIEGEL et al., 1986; KLOAS et al., 1999).

Sex reversal
Sex reversal has been induced in *X. laevis* by the administration of 17β-estradiol to genetically male larvae during stages 51-54 (VILLALPANDO and MERCHANT-LARIOS, 1990). RAMSDELL et al. (1996) were able to demonstrate the same response with exposure of *X. laevis* to nonylphenol. Several laboratories are presently aiming at developing this assay as a standard technique (e.g. KLOAS et al., 1999).

Recently US EPA (2002b) has published a revised draft Detailed Review Paper for amphibian metamorphosis assay.

*4.2.2.3 Invertebrate tests*

An in-depth understanding of life history, morphology, and the influence of environmental conditions on invertebrates is required to determine whether a chemical can impact endocrine-driven functions in a population of invertebrates (STAHL and CLARK, 1998). Knowledge on the underlying hormonal processes is needed to distinguish chemicals that interact with the endocrine system, from environmental conditions (including chemicals) that affect or inhibit hormonally regulated physiological processes via other mechanisms.

Crustaceans have a characteristic pattern of ecdysteroid concentrations during the course of the molt cycle, which could provide a marker of hormone function. If baseline patterns are established in control populations, the measurement of circulating levels of ecdysteroids, e.g. by radioimmunoassay (CHANG and O'CONNOR, 1979), could be a practical test for EDCs detecting perturbations in normal ecdysteroid levels.

The Crustacean hyperglycemic hormone (CHH), which can be measured by ELISA, may be a potential test for endocrine disruption. Exposure to some heavy metals and organic chemicals has shown to affect hemolymph levels of CHH (reviewed in FINGERMAN et al., 1998). However, it is not clear whether these chemicals directly target CHH synthesis and/or secretion or whether effects on CHH levels are the consequence of general physiological stress. Moreover, due to the fact that various environmental factors such as temperature, salinity, and hypoxia might affect CHH levels the interpretation of test results might be difficult (CHANG et al., 1998).

Promising candidates for developing EDC-specific bioassays are chronic reproduction tests with parthenogenic stages of various daphnid species that are used for regulatory purposes of chemicals (EPA, 1986 and 1989; OECD, 1992d; EPA, 1994). The biology of daphnids is well known and reproductive success, offspring morphology, and the switch from asexual to sexual reproduction (and *vice versa*) are potential endpoints for EDC-bioassays. Recently, a correlation was found between chemicals that adversely affect reproduction in chronic tests with daphnids and those that interfere with steroid metabolism in daphnids (LEBLANC, 1998).

The induction of imposex and altered steroid metabolism in different invertebrate species might have potential as markers of endocrine effects; for example tributyltin and testosterone have been shown to induce imposex (BETTIN et al., 1996). The mechanism of imposex induction has been hypothesised to be metabolic androgenisation, that is, elevated levels of endogenous androgenic hormones (BETTIN et al., 1996; RONIS and MASON, 1996; LEBLANC and BAIN, 1997).

From the broader perspective of environmental EDCs, disruption of endocrine systems controlled by ecdysteroids and farnesyl hormones in invertebrates should be examined (LEBLANC et al., 1999).

US EPA (2002c) has published a draft Detailed Review Paper on mysid life cycle toxicity tests in which a two generation toxicity test is recommended. Endpoints are survival, growth rate and reproductive output (time to first brood, clutch size, sex ratio). There is little experience with mysids as ecotoxicological test organisms in Europe. However the use of marine mysid species should be taken into consideration for WEA.

*4.2.2.4 Synopsis of in vivo screening methods*

Fish
Because of the great diversity of fish, it might be desirable to include several fish species representative of different taxa and environments for screening purposes. Despite the large amount of research with teleosts regarding endocrinology and reproductive toxicology, experience is lacking with specific assays for screening for endocrine activity, including standardisation of such assays. Short-term *in vivo* tests that

consider a suite of integrative endpoints (e.g. gross morphology, vitellogenin measurement and histology of gonads) are recommended as screening tools. Depending upon availability of resources, more endpoints could be added in this screen, including measurement of plasma steroid concentrations, steroidogenesis, hypothalamic-pituitary function, fertilisation success and sexual differentiation in offspring.

Amphibians
The frog embryo teratogenesis assay (FETAX) is an appropriate starting point to develop a screening test which would include mode of actions specific for EDCs.

Invertebrates
The sensitivity of developmental stages and critical periods of endocrine function in invertebrates exposed to potential EDCs are not yet known. Therefore, it is currently difficult to identify the specific life stages or endpoints that should be incorporated into tests for evaluating potential effects of EDCs. Direct and indirect evidence suggests that invertebrates are sensitive to chemicals in the environment that disrupt endocrine systems. However, from the perspective of screening for endocrine effects, uncertainty exists concerning the role of endocrine systems in invertebrates. Therefore, it is premature to recommend specific tests/endpoints. However, from the standpoint of existing tests, it is suggest that sexually reproductive stages be considered in the chronic reproductive and developmental toxicity.

## 4.3 Secondary testing methods

After having developed and validated appropriate screening tools to identify hazard of potential EDCs, secondary testing methods can be applied to specify endocrine effects. Results from secondary tests should be relevant to be used in established environmental risk assessments (ERAs) (e.g. EU 1993a, b).

### *4.3.1 Fish tests*

As for the *in vivo* screening methods the fish tests described in the DRP (OECD, 1997) were the starting point for the OECD Expert Consultation (OECD, 1999 and 2000) to propose secondary testing methods allocated to two tiers of testing.

At the lower tier two test methods were recommended, which are complementary and allow to compare sensitivities of the developmental and reproductive endpoints. As developmental test an enhanced Fish Early-life Stage Toxicity Test (OECD, 1992b) and as terminal reproductive test a partial life-cycle test was proposed.

The rationale for exposing early life stages is that these are very sensitive stages. In the case of endocrine-active substances, sexual differentiation that is fully hormone-dependant is known to be the most critical period. Whether exposure includes the embryo-larval stages and whether a growing-period is required, will depend on the biology of the fish species. The endpoints measured in the Fish Early-life Stage Toxicity Test (OECD, 1992b) are hatching success, abnormal appearance and behaviour, body weight and length, embryo-larval development and deformities. With respect to selection of endpoints for detection of endocrine disrupting effects, it was suggested to concentrate on effects, which are specifically related to reproduction since effects on other endocrine effects (e.g. thyroid effects) are not yet well understood. Enhancements and additional endpoints compared to OECD (1992b) should be that exposure should cover sexual differentiation and early gametogenesis; furthermore gonadal histology should be examined at completion of sexual differentiation. Optional enhancements and optional additional endpoints could be post-exposure of fish (without contaminant) for measurement of fecundity and the determination of biomarkers (e.g. vitellogenin induction, sex steroids). An example for an enhanced Fish Early-life Stage Toxicity Test is given by TYLER et al. (1999).

In the partial fish life cycle test the reproductive performance, fertility of adult fish and mating behaviour

can be studied in addition to the effects covered by OECD (1992b). Exposures should start with sexually mature adult fish held in mating groups and might last for several weeks. The main endpoints are time to first spawning, spawning frequency, number of eggs per batch or spawning event, number of eggs per female, number of fertilised eggs, hatching success. As optional endpoints biomarkers (e.g. VTG induction) and histopathology of endocrine organs can be determined.

There is a need for research and comparative testing of the terminal reproductive test and the enhanced early life stage test. For some chemicals it might be possible that only one or the other test is necessary, while other substances might have to be subjected to both tests of the lower tier.

At the higher tier, the Fish Full Life-Cycle Test is recommended as a confirmatory test. The EPA Standard Evaluation Procedure OPPTS 850.1500 (EPA, 1996b) is considered to be a good starting point and can be used as basis with appropriate enhancements. In addition to fathead minnow, extension to other species (e.g. zebrafish, medaka, guppy) is strongly desirable but may be technically challenging due to problems in larval survival and juvenile hermaphroditism or in-breeding depression. The following endpoints should be measured: hatching and viability of the embryos ($F_0$ and $F_1$); larval survival, growth and development ($F_0$ and $F_1$); time to sexual maturity ($F_0$); secondary sexual characteristics ($F_0$); sex ratio ($F_0$ and $F_1$); egg production ($F_0$); spawning frequency and behaviour ($F_0$); fertilisation success ($F_0$ and $F_1$); gamete maturation ($F_0$). As optional endpoints biomarkers (e.g. VTG induction, sex steroids, steroid enzymes in $F_0$ and $F_1$) and histopathology of endocrine organs can be determined.

It should be emphasised that basic research is required to better define the developmental stages of various fish species as well as to identify suitable species to be used for assessment of gonadal development.

### *4.3.2 Invertebrate tests*

Ecological risk assessments are driven by evidence of effects rather than mechanistic data, therefore adverse effects on whole-organism endpoints (e.g. development or reproduction) represent a greater level of potential ecological risk than does mechanistic evidence of endocrine activity (PINDER and POTTINGER, 1998).

There is not sufficient information to identify those invertebrate species that have the greatest promise of detecting EDCs and that can be used to provide a sufficient degree of protection for other invertebrate species in the environment. It is proposed that a subset of surrogate species should be identified for testing. The results obtained from these tests could be used in ERA schemes to protect a range of species.

Based on the knowledge of endocrine systems in invertebrates the endpoints survival, fecundity, sexual maturation, sex ratio, mating behaviour, biomass, growth rate, molt time and success, embryonic development, larval development, and feeding behaviour should be selected to study the potential for effects caused by altered endocrine function. The endpoints selected are not necessarily exclusively responsive to an endocrine mechanism of action but should be used with more specific, diagnostic testing in a weight-of-evidence approach to identify endocrine disruption as the mechanism of action.

Invertebrate organisms of the aquatic environment requiring limited method development to conduct full life-cycle or transgenerational exposures are rotifers, nematodes, polychaetes, oligochaetes, amphipods, *Artemia* spp., cladocerans, copepods, mysids and grass shrimp.

Once a set of species and endpoints is identified, standard methods should be developed. Revisions of existing standard methods or the development of new standards to address effects of EDCs should not be finalised until research has been conducted to evaluate relative endpoint and species sensitivity. While most of these methods are likely to be whole-organism tests, *in vitro* screening methods should be developed and compared with whole-organism testing to help in the screening process as well as to

support mechanism of action studies (DEFUR et al., 1999).

## 5. RELEVANCE OF TEST METHODS FOR WASTEWATER DISCHARGES

In many scientific studies it has been demonstrated that effluents from wastewater treatment plants (WWTPs) across Europe cause estrogenic effects in fish when using VTG as biomarker (HANSEN et al., 1998; SHERRY et al., 1999; SOLÉ et al, 2001). GAGNÉ et al. (2001) used the freshwater mussel *Elliptio complanata* to evaluate estrogenic effects of WWTPs. Furthermore, the reporter gene assay with recombinant yeast cells has been used by several researchers (e.g. ZACHAREWSKI et al., 1995; ROUTLEDGE and SUMPTER, 1996; TANGHE et al., 1999; REHMANN et al., 1999a, REHMANN et al., 1999b) to screen aquatic environmental samples for estrogenic activity. However, this assay is limited to detect one MOA of endocrine substances. The potency of estrogenicity varies widely (HARRIES et al., 1999; LARSSON et al., 1999; PURDOM et al., 1994). Sexual disruption has been shown to occur in wild populations of roach (*Rutilus rutilus*) (JOBLING et al., 1998) and gudgeon (*Gobio gobio*) (VAN AERLE et al., 2001), showing that the problem is not species-specific. In a study on the flounder (*Platichthys flesus*), incidences of intersex fish were observed in the marine environment (TYLER, 2001). Intersex fish have also been identified as a consequence of exposure to effluents from WWTPs in France (FLAMMARION et al., 2000; MINIER et al., 2000), Sweden (LARSSON et al., 1999), Germany (HANSEN, 2000) and Denmark (CHRISTIANSEN et al., 2000). Studies in the United States of America have also reported sexual disruption in caged and feral fish exposed to effluents from WWTPs (FOLMAR et al., 1996). Furthermore, evidence for sexual disruption has been shown to occur in flatfish (*Pleuronectes yokohamae)* around the coast of Japan (HASHIMOTO et al., 2000). In the United States of America, not all sexual disruption reported in fish occurred as a consequence of feminising effects. For example, Mosquito fish *(Gambusia affinis)* exposed to effluents from pulp mill effluents have been reported to be androgenised (BORTONE et al., 1989). Differences in the level and severity of endocrine disruption in different wildlife populations is likely to depend not only on their level of exposure to EDCs, but also on differences between species in their sensitivity to chemicals.

OSPAR (2000) has compiled and described standardised, approved and proposed methods for testing of wastewater. These methods have not been designed to identify specific effects related to endocrine systems of aquatic vertebrate and invertebrate organisms. Yet, the following overlap can be identified when comparing the list of methods for testing of wastewater (OSPAR 2000 Annex I-3) with the testing methods described in the previous sections of this Draft background document:

- The long-term fish toxicity tests for adult fish (OECD, 1984; OSPAR 2000 Annex I-3) used in testing of wastewater is recommended to be the basis for one out of two proposed *in vivo* screening methods for evaluating potential effects of EDCs.
- The long-term fish toxicity test (Fish Early-life Stage Toxicity Test: OECD, 1992b; OSPAR 2000 Annex I-3) used in testing of wastewater is considered to be the starting point for the development of one out of two recommended lower tier secondary testing methods to determine environmental risks of EDCs. Thus data derived from secondary testing methods might be useful for whole effluent assessment.
- The test organism of the long-term *Daphnia magna* Reproduction Test (OECD, 1998; OSPAR 2000 Annex I-3) used in testing of wastewater might be one of several surrogate species representing the invertebrate animals when identifying and measuring endocrine effects to be used in environmental risk assessment schemes. However, it should be emphasised that basic research is required to identify those invertebrate species that have the greatest promise of detecting EDCs and to understand the mode of action of EDCs in invertebrates.

In general, discharges to fresh water and salt water containing chemicals that disrupt endocrine systems are more likely to be detected in those countries that incorporate sublethal endpoints in long-term tests

(LEBLANC et al., 1999). Therefore, it is recommended to incorporate long-term methods for ecotoxicity testing in WEA which increases the probability to determine endocrine effects in effluents. The experience gathered so far reveals that short-term ecotoxicity testing is unlikely to measure relevant endocrine effects in effluents.

# 6. CONCLUSIONS

General aspects of endocrine disruption:

- Through the endocrine system organisms regulate principal life functions such as growth and development, homeostasis, behaviour, reproduction and the immune system.
- Within the endocrine system endogenous hormones serve as the chemical messengers between cells and organs.
- Endocrine-disrupting chemicals can copy or corrupt the hormonal messages and cause adverse health effects in organisms or their progeny.
- The endocrine systems in invertebrates differ considerably from those in vertebrates.
- Various modes of endocrine disruption can be identified but only one of these, namely estrogenicity, has been intensively studied in the past decade.

Estrogenicity:

- Several *in vitro* and *in vivo* test methods have been developed in order to detect the estrogenic potency of chemicals, but up to now only draft standard test procedures have been established.
- A small number of *in vitro* tests, mainly those based on hormone receptor activation (e.g. YES assay) and human breast cancer cell line proliferation (e.g. E-Screen) have been applied to wastewater samples. *In vitro* tests are generally time and cost-effective, but since they cover only single cellular or sub-cellular endpoints, they have limitations as predictive tools in environmental hazard risk assessment (high risk for false negatives).[1]
- Endpoints such as gross morphology, histopathology of the gonads and induction of vitellogenin in fish and amphibians have been successfully used in non-standardised *in vivo* tests for wastewater assessment. Despite their predictive power, however, the considerable amount of effort limits their applicability for routine measurement.
- Currently no single standardised and validated test system, which detects specific endocrine-disrupting effects can be recommended for wastewater evaluation. The development of suitable test systems is going on and OSPAR should observe this field over the next two years. It is likely that the OECD and other bodies will propose *in vivo* tests for the testing of chemicals that can provide more and varied measurable effects of endocrine disruption including those expressing reproductive and transgenerational effects. Work on standardising *in vitro* tests has been started.
- At the moment it is recommended to incorporate chronic (partial life cycle and/or reproduction) methods for ecotoxicity testing in WEA since these tests will reduce the probability of leaving effluents with endocrine disrupting effects unnoticed.[2]

[1] This conclusion does not necessarily express the view of all Contracting Parties.

[2] This recommendation does not necessarily express the view of all Contracting Parties.

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