Global Water Research Coalition

Endocrine Disrupting Compounds

An overview of sources and biological methods for measuring EDC

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IWA affiliate

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Global Water Research Coalition: Cooperation for worldwide water knowledge, innovation and progress

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 members of the GWRC are: the Awwa Research Foundation (US), CRC Water Quality and Treatment (Australia), Kiwa (Netherlands), Sues 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), WaterReuse 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 organisations 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 US Environmental Protection Agency a partnership agreement was signed in July 2003.

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An overview of the sources and biological methods for measuring EDCs

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Contents

Preface

The possible effects endocrine disrupting compounds (EDCs) in water systems have been the subject of considerable attention by a wide range of different groups including regulatory agencies, researchers, industry, water utilities, NGOs and the media.

EDCs were identified by the Global Water Research Coalition (GWRC) as the first research priority that was of common interest to all members. The research agenda of the GWRC includes the preparation of a report that will give an overall view of the current position regarding EDCs in water systems. To achieve this end workshops on the occurrence and fate of EDCs (WRC, South Africa, 2002) and analytical methods and bio-assays (Water Technology Center, TZW, Germany, 2003) were held in order to collect information available to the GWRC-members on this topic.

This review is a brief overview of the sources, occurrence and biological methods for measuring EDCs in the aquatic environment and is prepared within the framework of the joint research programme of the Dutch drinking water companies.

1 Introduction

Water can act as a sink for a variety of hazardous substances that arise in discharges, and by run-off and other diffuse sources, including wastewater from cities and towns. Consequently, the occurrence of hazardous substances in water is an issue of concern at the global scale. The reduction of emissions of toxic, persistent or bio-accumulating substances is an agreed goal of a number of international organisations and this has, in turn, been incorporated in the policy of many countries. The objectives of such policy are to protect both aquatic life and human life, which can be exposed directly, or more frequently, indirectly to such substances. One of the more recent concerns to emerge has been that of Endocrine Disrupting Chemicals (EDC) in the environment [Colborn, 1996; Keith, 1997]. A number of international workshops have been held in order to explore this topic and much of this interest has focussed on water, for example both IWA (Melbourne, 2002) and AWWA (Cincinnati, 2002) have held workshops on EDCs.

A number of initiatives are in progress in different parts of the world to determine the impact of EDCs and, if necessary, how controls can be put in place. The subject continues to engage a wide range of stakeholders, including regulators, industry, water utilities, environmental NGOs and researchers. These groups hold a variety of views but all recognise the need to determine the hazard, assess the risks and to determine the most appropriate solutions.

The endocrine system is a key control system of the body operating through glands that secrete hormones, which act as chemical messengers. Hormones are secreted directly into the bloodstream and interact with specific receptors on cells that enable functions to be switched on and off. The endocrine system is highly complex, particularly in higher animals, and interference by foreign chemicals could lead to serious consequences, particularly if changes occur in the development of the embryo or the young animal. An endocrine disrupter could be defined as ‘a substance that causes perturbation of hormonal/endocrine status at dose levels which are not cytotoxic.’ In other words, substances that cause changes in endocrine status only at doses that also cause tissue damage would not be considered. Although a wide range of potential endocrine systems could be considered the one that has received most attention to date is the reproductive system and, particularly, estrogen. It is important to remember that man has always been exposed to exogenous substances that possess estrogenic activity through his food, in particular phyto estrogens. However, the first indications that there may be a concern for water arose as a consequence of the identification of changes in male fish downstream of sewage treatment plants, which were subsequently shown to arise following exposure to natural human and synthetic hormones that had survived the sewage treatment process.

This was subsequently confirmed in many inland waters downstream of effluent discharges from sewage treatment plants and there are also data indicating such activity in estuarine and inshore marine fish species. Studies carried out in the United States, Canada, Europe, Japan, Australia and South Africa, show that over 50 substances suspected of possessing endocrine disrupting properties may be present in water used as a source for drinking water supply [AWWA, 2002].

Other effects in wildlife associated with the aquatic environment that have been attributed to EDCs are reproductive problems in animal populations, such as alligators and fish-eating birds, and feminization of male birds, turtles and alligators. These effects were shown by the presence of the female specific egg yolk precursor protein, vitellogenin, in males, ovotestis in males, development defects in the sexual organs (intersex) and disturbance of sex ratio's in affected populations [Mukerjee *et al*., 2002, Vethaak *et al*., 2002]).

Low concentrations of some industrial chemicals and natural and synthetic hormones may affect and cause damage to hormone systems and disturb the natural balance in eco-systems, wildlife and possibly humans. EDCs can mimic hormones or may block the activities of hormones. It may also be possible that some EDCs are promoters or inducers of some type of cancer such as breast cancer [Keith,1997].

Chemicals that have been shown to possess estrogenic activity, and in some cases to be capable of disrupting the endocrine system in intact animals include the following categories: natural and synthetic hormones, phyto-estrogens, some pesticides, some organic solvents, alkylphenols and alkylphenol-ethoxylates, bisphenol A, phthalates, organohalogen compounds such as polychlorinated- and –brominated-biphenyls, -diphenylethers and dioxins, and some metals [Colborn, 1996; WWF, 1996; EPA, 1996]. For many chemicals the potential to impact on endocrine activity has not been investigated , therefore wider screening for ED activity using bioassays is needed to assess and monitor the potential hazard of these chemicals.

There is also a need to more clearly identify the substances responsible for estrogenic activity in water since the total estrogenic activity observed in many types of water cannot be explained completely by the measured concentrations of natural estrogenic compounds [Lévi *et al*., 2002, Vethaak *et al*., 2002].

This review gives a brief overview of the substances that are suspected of possessing endocrine disrupting activity, examines the sources and routes to water, briefly considers the occurrence in the water cycle, examines potential bioassays for measuring integrated estrogenic activity and identifies some research needs.

2 Effects of EDCs

Assessment of effects of EDCs is very complex since exposure may be to multiple chemicals with the potential for synergistic, antagonistic and additive effects.

Effects on human health ascribed in the literature to EDCs are reproductive disorders, inhibition of female characteristics, breast cancer and testicular cancer, decreasing semen quality, increasing frequencies of undescended testes and hypospadias and an increased demand for assisted reproduction [Bornman, 2000]. In recent years, apart from direct effects on hormone dependent tissues, other indirect effects have come under consideration. Theses include effect of EDCs on the development of the brain either in utero or of the very young which may possibly lead to structural alterations or behavioural effects which would only be observed at a later date.

As indicated above a number of effects have been recorded in wildlife, including feminization of males alligators, birds and fish [Mukerjee *et al*., 2002].). Sexual abnormalities in the sexual organs of juvenile male alligators in Florida have been associated with the presence of high levels of EDCs . The presence of ovotestis and the production of vitellogenin, the female egg yolk protein produced in the liver under the control of estrogen, has been reported in male fish in the UK and the Netherlands. Poor breeding success in Western Gulls was reported in California as being associated with the presence of EDCs.

2.1 Substances with known EDC effects

There are different classes of chemical compounds which are known EDCs or suspected of having endocrine effects. Different testing systems are used to test chemical compounds for their potential effects: these testing systems are based on mechanisms such as binding to the estrogen–receptor, initiating transcription activity via mRNA synthesis and subsequent protein synthesis, or the influence on cell growth shown in specific estrogen sensitive breast cancer cell lines. However, in order to be demonstrated to be an endocrine disrupter in intact animals, in vivo studies are also required.

One of the short-term actions of the European Commission, following the European Community Strategy for Endocrine Disruptors, is the establishment of a priority list of substances for further evaluation as to their role in endocrine disruption. During 2000, a candidate list of 533 man-made substances and 9 synthetic or natural hormones was identified. The candidate list was divided into three separate groupings of substances depending on the level of information available, an a priority list of actions was developed. For 12 substances, including two hormones, oestrone and oestradiol, and one synthetic hormone, ethinyloestradiol, there is scientific evidence of endocrine disruption or potential endocrine disruption. It is not possible to take action regarding these substances at source. 115 Substances have been selected for which there is evidence of endocrine disruption or potential endocrine disruption, and which are already regulated or are being addressed under existing legislation. For 435 substances insufficient data were available to decide on whether they were potential for endocrine disrupters [European Commission, 2001].

The classes of chemical compounds for which potential endocrine effects can be identified are as follows[European Commission, 2001]:

- Natural hormones: estrone, 17a-estradiol, 17ß-estradiol, estriol, testosterone, progesterone, trenbolone, zeranol;
- Synthetic hormones: diethylstilbestrol (DES) , norgestrel, mestranol, melengestrol-acetate, lynestrenole, 17a-ethynylestradiol;
- Phyto-estrogens: coumestrol, genistein;
- Dioxins and furans: 2,3,7,8-TCDD;
- Hexachlorobenzene(HCB), polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and polybrominated diphenylethers (PBDE);
  Phthalates: butylbenzylphthalate, bis(2-ethylhexyl)phthalate, dibutylphthalate, diethylphthalate, (some phthalates esters showing anti-androgen effects);
- Alkylphenols and alkylphenol-ethoxylates: 4-tert-octylphenol, 4-n-octylphenol, 4-n-nonylphenol;
- Pesticides: acetochlor, alachlor, aldicarb, aldrin, amitrole, atrazine, carbofuran, chlordane, 2,4-D, deltamethrin, dieldrin, endosulphan, endrin, heptachlor, lindane, linuron (anti-androgen), DDD, DDE, DDT, maneb, metam-natrium, methoxychlor, nitrofen, parathion, simazine, 2,4,5-T; thiram, toxaphene, vinclozolin (anti-androgen); zineb;
- Organic chemicals: bisphenol-A (BPA), styrene, 4-nitrotoluene, n-butylbenzene, butylated hydroxyanisole, resorcinol, ibromochloropropane; n-4-dichlorophenol, 4-chloro-3-methylphenol, 2,2'-bis(4-(2,3-epoxypropoxy)phenyl propane, o-phenylphenol and carbondisulphide;
- Organotincompounds: tributyltin and triphenyltin and their degradation products di- and mono-butyl and phenyltin;
- Heavy metals: cadmium (Cd) and mercury (Hg).

3 Sources of EDCs and emission routes

Municipal wastewater and effluents from sewage treatment plants constitute an important source of contamination of surface water with natural and synthetic hormones (estrone, 17ß-estradiol, 17a-ethynylestradiol, estriol). In some cases, xeno-estrogens like alkylphenols and alkylphenol-ethoxylates are also present in significant amounts since these compounds are widely used as non-ionic surfactants. Although concentrations are reduced significantly during treatment the effluents can be highly estrogenic to male fish, for example by inducing the production of high levels of vitellogenin, the egg yolk protein, in male rainbow trout (VTG-induction),[Vethaak *et al*., 2002].

Many plants e.g. legumes contain phyto-estrogens, such as isoflavenoids. These compounds are common in human and animal food. Isoflavones are found in most plant tissues and include the estrogenic compounds genistein, diadzein, biochanin A and formononetin , [Adlercreutz *et al*., 1998].
Manure from cattle is another source of natural hormones since dairy cows, in particular, excrete significant amounts of estrogens and other hormones in their urine. However, very few data are available on emissions to water from this source.

Wastewater from industries may be an important source of synthetic EDCs (bisphenol-a, alkylphenols, phthalates, polybromobiphenyls and polybromodiphenyl-ethers). The use of pesticides in agriculture, like atrazine, lindane and other plant protection products suspected of possessing ED activity, is a major sources of diffuse contamination of surface water and groundwater.

Since estrogenic activity and xeno-estrogens have been measured in rainwater (phthalates), a contribution from rainwater to the occurrence in surface water cannot be excluded.

Dioxins are of significant concern as potential endocrine disrupters but the main sources of dioxins in industrial countries are emissions from waste incineration and the metal industry. The main source of exposure of humans to dioxins is from fish, meat and dairy products [Bornman, 2000]. Polychlorinated biphenyls (PCBs), some of which possess dioxin-like characteristics, are widely found in the environment as a consequence of their past use in electrical equipment. Much of the PCBs found in the environment arise as a consequence of historical pollution from poor disposal of electrical transformers. Human exposure to PCBs is mainly from food (fish, meat, milk products, breastfeeding).

Organotin compounds can migrating in surface water from anti-fouling paints applied to ships and boats. These compounds are now banned in many countries.

3.1 Priority EDCs in wastewater

UKWIR have carried out a review to identify EDCs of particular importance with respect to their occurrence in wastewater [UKWIR, 2001]. The selection of chemicals was largely based on the UK Environment Agency's strategy on endocrine disruptors in the environment, launched in 2000 [Environment Agency, 2000] This strategy identified a number of chemicals that would require priority action in terms of reducing

their emissions to the environment. Further evaluation of these was undertaken in order to identify those which were of particular importance both in terms of a) their endocrine disrupting activity and potential environmental impact and b) their likely presence in domestic sewage. Potential future European and scientific developments were also considered to identify whether additional chemicals should also be included. The chemicals considered were: steroid oestrogens, alkylphenol polyethoxylates and their breakdown products, phthalate esters, bisphenol-A, certain pesticides and PCBs.

On the basis of this review UKWIR concluded that steroid oestrogens are the most likely candidates for future regulatory control. Firstly, they have been demonstrated in several studies to be the more estrogenically potent components of domestic sewage effluent. Secondly, a growing number of studies in fish have been published which support the view that they cause oestrogenic effects in fish at concentrations of a few nanogram per litre. Since the compounds are excreted naturally by humans and cannot easily be controlled at source, emissions to the environment from sewage water treatment systems will be dependent in part upon the degree of treatment given.

Alkylphenols and short-chain ethoxylate metabolites, derived from the widely used alkylphenol polyethoxylates products, represent another group of compounds of particular interest. Although of relatively low oestrogenic potency their occurrence is widespread and some localised impacts have been noted.

Both phthalates and bisphenol A may be found in sewage effluent at low concentrations but, given their weak oestrogenic potency, they are currently of low concern as endocrine disrupting chemicals. Nonetheless, in view of possible future regulatory interest, they have been included in the subsequent review.

Some pesticides, PCBs, and anti-foulant compounds have been shown to have weak oestrogenic properties. However, their occurrence in domestic sewage is relatively low and they would be expected to contribute more through diffuse inputs into the environment.

4 Occurrence of EDCs in the water cycle

Data on the occurrence of EDCs in water systems were collected in 2002 by means of a questionnaire sent to the members of the GWRC. The results of this inventory were evaluated at a GWRC-workshop in South Africa and described in a subsequent GWRC-report [Burger *et al*., 2003]. This summarizes information from the United States of America, Canada, South Africa, Australia, United Kingdom, France, Germany, and the Netherlands.

The conclusion from the evaluation of these data is that natural and synthetic hormones and xenoestrogens are present in all countries but in decreasing concentrations in influents and effluents of sewage treatment plants and the receiving surface water. EDCs have also been measured in low concentrations in rainwater and drinking water and activity has been detected with bioassays, described in more detail in chapter 4 (ER-CALUX®).

The estrogenic activity measured with the ER-CALUX® assay in effluents of wastewater treatment plants were in the range of 4 to 440 pmol/l EEQ (estradiol equivalents). For effluents the values varied between 0.11 - 59 pmol/l. Water extracts from four large rivers in the Netherlands showed levels ranging from 0.25 to 1.72 pmol/l. Extracts, from suspended matter and sludge, contained estrogenic activity of 0.26 to 2.49 and 1.6 to 41 pmol EEQ/g dry weight, respectively. In waste water treatment plants (WTPs) the average reduction of estrogenic potency in effluent compared to influent was 90 – 95 % in municipal WTPs and about 50% in industrial WTPs. In influent, 30 % of the ER-CALUX® activity could not be explained by the calculated activity based on chemical analysis of a number of known (xeno-) estrogens in effluents. In effluents the unexplained fraction was 80%, [Murk *et al*., 2002].

In drinking water the ER-CALUX® activity, measured after isolation of organic compounds from the water sample with solid phase extraction (SPE), was in the range of 0.005 - 0.74 pmol/l. Most of the values were below the limit of quantification (< 0.003 pmol/l). There was a significant reduction in activity during drinking water treatment processes, compared to the ER-CALUX®-values measured in surface water, [Ghijsen and Hoogenboezem, 2000].

5 Bioassays and biomarkers

5.1 Introduction

A wide range of chemicals has been reported to have estrogenic activity. The application of bioassays is therefore a valuable tool beside chemical analysis of target EDCs, to measure the total activity (estrogenic or androgenic) resulting from all of the chemicals present, including any unknown EDCs.
The assessment of EDC activity is made by in vitro and in vivo methods. In vitro methods are often based on the interaction of an EDC with the endocrine system at the cellular level using cell cultures. Most of these test are based on the binding of EDC to a specific receptor on a test cell.
Effects in in vivo tests are measured in the whole animal by a number of possible endpoints such as an increase in uterus weight. Although in vivo experiments have the great advantage of taking into account absorption, metabolism and excretion and being of direct relevance, they can be time-consuming and costly.

Many techniques are described in the literature to measure the activity of EDCs. This chapter reviews the principles of some of these methods and the reported sensitivity (lowest observed effect concentration and measured ranges) for application in different types of water: influents and effluents of wastewater treatment plants (WTP), surface water, groundwater and drinking water.

5.2 In VITRO assays

5.2.1 Estrogen Receptor (ER) binding assay

This assay used for screening the estrogenic potency of water samples is described by Murk *et al*., (2002) . This test examines the binding capacity of a compound to the estrogen receptor (ER). The sensitivity of this assay expressed as the 50 % response (EC_{50}) amounts 6-9 nM 17ß-estradiol. In this assay both agonists and antagonists give an estrogenic response resulting in higher 17ß-estradiol equivalency (EEQ) levels for water samples compared to the YES- and ER-CALUX® assay (see 6.2.2- and 6.2.4).
Quanrud *et al.* [2002] concluded that the ER- binding assay is the least physiologically significant, least sensitive test, and tends to show higher concentrations of estrogenic compounds in water samples than the other tests. Reasons for these reported higher concentrations are that the test additively detects both estrogens and anti-estrogens and that the test does not account for the barriers for transporting the chemicals across the cell wall.

5.2.2 Enzyme linked immun-assay (ELISA)

An ultra-sensitive immunoassay has been developed and tested for the quantisation of 17ß-estradiol at low levels (1.5 ng/L) in water samples without any sample pre-concentration [Rubio *et al*., 2002]. The precision varies between 2 and 16 %. The specificity is good, other hormones show a cross-reactivity ranging from < 0.01 to 0.7 % (estrone 0.7%, estriol 0.3 %). The average recovery for 17ß-estradiol added to groundwater samples is 95%.

Interference by nitrate, magnesium, calcium, sulphate, phosphate was not observed at levels up to 20,000 ppm.

5.2.3 Yeast estrogen screen (YES) assay

The yeast estrogen screen (YES) [Routledge and Sumpter,1996] is a recombinant reporter gene assay that uses yeast cells stably transfected with human ER-a cDNA and an ERE-regulated expression plasmid (*lac-Z*). Interaction of an estrogenic compound with the estrogen receptor results in expression of the reporter gene *lac-Z* and secretion of the enzyme ß-galactosidase in the yeast medium. ß-galactosidase transforms the yellow substrate chlorophenol red–ß-D-galactopyranoside (CPRG) present in the medium to red. This is measured spectrophotometrically, [Vethaak *et al*., 2002]. The EC_{50}, that is the concentration causing 50 % of the maximum response, amounts 100 pmol 17ß-estradiol. Quanrud reported a EC_{50} of 6 – 9 nmol 17ß-estradiol [Quanrud *et al*., 2002].

Two-hybrid recombinant yeast cell bioassay TRCBA (Nishhikawa *et al*., 1999).
This bioassay is used in Japan to measure the estrogenic activity in surface water, process water and drinking water [Kobuke *et al*, 2002].
After filtration with glass fibre filters to remove solid particles, the water samples were then passed to a C-18 solid phase cartridge . Residual water was removed under a nitrogen stream. The adsorbed organic compounds were desorbed with 2 ml methanol. Estrogenic activity of the concentrated samples was determined by use of Two-hybrid recombinant yeast cell bioassay TRCBA [Nishikawa *et al*.,1999].

Estrogenic activity was measured in surface water and effluents of wastewater treatment plants. Poor elimination of estrogenic activity by biological treatment at the sewage treatment plant resulted in the occurrence of estrogenic activity in the source water of drinking water treatment plants. Coagulation, flocculation and filtration removed about 50 % of the estrogenic activity. Chlorination removed the estrogenic activity to the measured blank level.

Kawagoshi *et al*., [2002] simplified the original method described by Nishikawa and used the simplified method for screening estrogenic effects in leachate from a landfill. The sensitivity of the method was about a factor 10 times higher than the original method. EDCs were extracted from the water samples by extraction with dichloro-methane at pH 7 and pH 3 . The extract was concentrated nearly to dryness and dissolved in methanol. With this method the estrogenic activity of leachate was in the order of 0.1 nmol of 17ß-estradiol. The main contribution to the activity could be explained by measured concentrations of bisphenol A and alkylphenols.

5.2.4 E-screen cell proliferation assay

The E-screen MCF-7 cell proliferation assay is widely used to detect weakly estrogenic compounds. A dose-related increase in cell numbers in treated cultures is taken as evidence of estrogenicity of the test compound. MCF-7 estrogen-senitive human cancer cells are used and cell proliferation measured [Soto *et al*., 1995]. The estrogenic activity of a test compound is evaluated by determination of the relative effect to cell proliferation. The sensitivity, expressed as 50 % response (EC_{50}) amounts 1 pM 17ß-estradiol {Quanrud *et al*., 2002]. There is no standardized protocol

for this assay, and various test regimes and cell sub-lines have been used. As a result, considerable variability has been reported in published results.

5.2.5 ER-Calux® assay

The estrogen receptor (ER)-mediated '*Chemically activated luciferace gene expression*' (ER-CALUX®) bioassay (Legler *et al*., 1999) is a recombinant reporter gene assay which offers highly sensitive measurement of the trans-activation of the ER following exposure to (xeno-)estrogens. The ER-CALUX® comprises genetically modified $T_{47}D$ human breast adenocarcinoma cells containing endogenous ER-a and ER-ß. An ER-mediated luciferace reporter gene construct containing three estrogen response elements (EREs) was introduced stably and integrated in the genome of the $T_{47}D$ cells. Exposure of cells to (xeno-) estrogens results in diffusion of chemicals through the cell membrane, binding to the endogenous ER, activation of the receptor, and consequently, binding of the ligand-receptor complex to EREs present in the promotor region of the luciferace gene. Luciferace protein is then induced, and is easily measured by lysing the cells, adding luciferin substrate, and measuring photon production. The amount of light produced is proportional to the amount of ligand-ER binding which can be related to estradiol equivalents, EEQs [Vethaak *et al*., 2002]. The test is extremely sensitive and capable of detecting femtograms of estrogenic substances. This allows small sample sizes. Using 96-well test-systems allows rapid analysis of large numbers of samples.

However, before measuring the estrogenic activity, extraction techniques like liquid-liquid extraction or solid phase extraction (ESP) have to be used to isolate EDCs from the matrix.

The EC_{50} of the ER-CALUX® assay amounts 6 pmol 17ß-estradiol and is more sensitive compared to the YES assay and CARP-HEP assay.

5.2.6 DR-CALUX® assay

Derived from studying the toxicological mode of action of dioxins an in vitro bioassay has been developed as a screening method for the detection of dioxins and/or dioxin-like compounds.

The Dioxin Responsive Chemically Activated Luciferase Expression (DR-CALUX®) bioassay comprises a genetically modified H4IIE rat hepatoma cell-line, incorporating the firefly luciferase gene coupled to DREs as a reporter gene for the presence of dioxins and /or dioxin-like compounds. Cells that are exposed to dioxins and /or dioxin like compounds not only express proteins and enzymes that are, under normal circumstances associated with the DRE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-AhR binding and related to 2,3,7,8-TCDD toxic equivalents (TEQs).

The method is extremely sensitive and capable of detecting femtogram of dioxins. However, before applying this bioassay the dioxins have to be extracted from the matrix (water, suspended matter or sludge).

5.2.7 Carp-HEP assay

The carp hepatocyte (CARP-HEP) assay [Smeets *et al*., 2002] allows measurement of estrogenic activity in natural untransformed cells. It measures vitellogenin which is the precursor of yolk proteins and which is secreted by liver cells in response to exposure to estrogens. Vitellogenin is produced in liver cells of female oviparous vertebrates. The vitellogenin gene is also present in male oviparous vertebrates but under normal circumstances the estrogen levels are too low to induce vitellogenin production. However, exposure to estrogens can induce vitellogenin in males.

The CARP-HEP assay is less sensitive compared to the YES- and ER-CALUX®-assay, for example the EC_{50} for 17ß-estradiol is 100 nmol [Vethaak *et al*., 2002].

5.3 In VIVO assays

The OECD proposed the development of guidelines for testing the estrogenic effects in vivo with fish. Three types of tests based on an in vivo test were distinguished:
'Juvenile Growth Test' OECD 204 with vitellogenin induction as endpoint and histopathological examination of the gonads of the larvae at the end of the testing period;
'Early Life Stage test' OECD 210 with vitellogenin induction as endpoint and effects on development of the gonads;
'Fish full life cycle test'.

Since that time different tests have been developed with a range of different fish species: (transgenic) Zebra fish, Fathead Minnow and Japanese Medaka. In situ exposure studies with rainbow trout, salmon, carp, bream and flounder have been successfully carried out for the examination of estrogenic activity.

Genetic differences in sensitivity to endocrine disruption by estrogen have been reported. Bioassays using selected animals may underestimate the potential effects in sensitive populations. These differences in sensitivity must be accounted for when a specific animal model is used for EDC effects, [Bornman, 2000].

Estrogenic effects in fish:
Hatching and survival of embryo's, larvae and (juvenile) fish in ELS-test;
General condition: condition factor for liver weight and gonad weight;
Sex ratio of fish in ELS-tests and wildlife;
Ovotestis in male fish;
Vitellogenin production measured in blood plasma of male fish or in whole body homogenates.

Vitellogenin production in serum of male Mature Medeka has been used to evaluate the estrogenicity of EDCs such as nonylphenol (NP), bisphenol-A (BPA) or 17ß-estradiol (E2). [Tabata *et al*., 2001]. Vitellogenin was not detected in 53 negative controls (exposure time 0 - 5 weeks, except in three cases: levels of 330, 889 and 3150 ng E2/ml). Test solutions of 100 µg/l p-NP, 1000 µg/l BPA and 0,05 µg/l E2 showed vitellogenin production. The relative potencies decreaed in the order E2 >> p-NP >> BPA. In the same experiments after chlorination of these test solutions for 24 hours the estrogenic activity was reduced drastically [Kobuke *et al*., 2002].

High concentrations of vitellogenin have been found in male bream together with a high number of ovotestis (33-43%) in surface water in the Netherlands [Vethaak *et al*., 2002]. This surface water receives effluents of domestic sewage treatment plants, but is also influenced by industry and agriculture.
Laboratory tests with zebrafish confirmed the estrogenic activity measured in effluents with the 'in vitro' ER-CALUX® test.

6 Recommendations

6.1 Effects of EDCs

There is a need for a list of all of the effects associated with endocrine disruption in relation to human health , effects on wildlife and ecotoxicological effects.

More information is needed on endocrine disruption in relation to effects on both human health and wildlife in relation to specific substances, low doses and mixtures of chemicals.

Currently, ecotoxicological information concerning potential reproductive effects in aquatic organisms is available only for a relatively small number of chemicals. It would be desirable to carry out experiments on the effects of EDCs on fish and other aquatic organisms [Vethaak *et a*l, 2002].

There is still a need for a testing strategy and a battery of validated testing methods to evaluate the potential endocrine disrupting activity of chemicals and to determine possible effects in aquatic ecosystems and human health risks from drinking water.

In order to decrease emissions of EDCs to the environment, more knowledge is needed about the fate of EDCs in wastewater treatment processes.

6.2 Monitoring

To define monitoring programmes, proper evaluation of available techniques is needed and research, development and validation requirements need to be identified.

Since estrogenic activity and target-EDCs have been measured in all parts of the water cycle (wastewater, surface water from ditches, canals, rivers, vulnerable groundwater, infiltrated water, rainwater, drinking water) data of the occurrence of EDCs need to be collated and used to assess the risks associated with these compounds.

Since, in some cases, the estrogenic activity measured in surface water and WTP-effluents with bioassays like the ER-CALUX® could not be completely explained by chemical analysis of known EDCs these in vitro test should be considered for use monitoring the potential activity of EDCs. The ER-CALUX® seems to be the most sensitive assay for screening the estrogenic activity together with pre-screening with the much cheaper ER assay.

For the evaluation of the predictive value of in vitro tests like the ER-CALUX® bioassay, comparative studies with in vivo tests using fish (transgenic zebra fish assay and ELS/PLC assays)or other organisms is necessary.

Together with bioassays for screening for estrogenic activity the following chemical parameters need to be included in monitoring studies [Vethaak *et al.*, 2002]:

- Hormones like estrone, 17ß-oestradiol, and 17a-ethynylestradiol;
- alkylphenols as nonylphenols and nonylphenol-ethoxylates (especially in suspended matter, sludge and sediments);
- Other suspected (potential) EDCs that are found frequently in surface water including phthalates, bisphenol A and pesticides;
- Hydrophobic EDCs like PCBs, PBBs, organochlorine pesticides, dioxins and organotin compounds although it would be more appropriate to restrict monitoring to suspended matter, sediments and sludge.
- To evaluate the risks of EDCs, correlations need to be made between the results of bioassays and concentrations of contaminants in fish or other wildlife (mussels), suspended matter, sludge, sediments and the water phase.

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