# CSTEE Opinion on Human and Wildlife Health Effects of Endocrine Disrupting Chemicals, with Emphasis on Wildlife and on Ecotoxicology Test Methods

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Report of the Working Group on Endocrine Disrupters of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of DG XXIV, Consumer Policy and Consumer Health Protection

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## Executive summary

### Background

There is growing concern on possible harmful consequences of exposure to xenobiotic compounds that are capable of modulating or disrupting the endocrine system. This concern for endocrine disrupting chemicals is directed at both wildlife and humans. Alteration of endocrine function caused by an endocrine disrupter may be through interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour. Industrial chemicals, consumer chemicals and chemicals in the environment can be endocrine disrupters that mimic, enhance or inhibit the action of hormones. Several expert working groups have concluded that there is increasing evidence of adverse effects in human and wildlife reproductive health, and have discussed the hypothesis that chemicals in the environment have caused these endocrine mediated adverse effects. This has led a series of stakeholders, including the European Commission, to consider the topic of endocrine disruption as of sufficient concern to justify action.

This led to the setting up of a Working Group on Endocrine Disrupters under the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE). The emphasis of this report is a review of the existing literature and scientific opinion on the evidence for chemically-induced endocrine disruption, in particular as it relates to the European wildlife, on EU testing strategy, on ecological risk assessment and on toxicological test guidelines. In addition, gaps in knowledge are identified and recommendations for future research made.

### Human health effects

Although there are associations between endocrine disrupting chemicals, so far investigated, and human health disturbances, a causative role of these chemicals in diseases and abnormalities possibly related to an endocrine disturbance has not been verified. At present the following conclusions can be made:

- a meta-analysis of 61 studies has reported a general decrease in sperm concentration and semen volume from 1938 to 1990. However, several reanalyses of the same data have indicated possible bias and confounding in the meta-analysis, and have reached different conclusions with respect to sperm quality, depending on the methodology used. Recent, well designed studies have shown that there are large regional differences in overall sperm quality and time trends, both within and between countries.
- for the reported increased prevalence in cryptorchidism or hypospadias no causative role for endocrine disrupting chemicals has been determined.
- the incidence of testicular cancer has increased significantly during the last 30 years. The underlying reason(s) for the increased incidence in testicular cancer has not been identified.
- there also has been recorded an increased incidence of prostate cancer in Europe during the last decades. Any causative role for xeno-oestrogenic chemicals in development of prostate cancer has not been established.
- there has been a steady increase in breast cancer incidence rates over the last decades in Europe. The available data associating breast cancer development with exposure to organochlorines do not support a causal relationship.
- there have been several reports on the declining proportion of male new-borns during the last decades; this decline in sex ratio remains unexplained.
- high accidental exposure to PCBs/PCDFs of pregnant women have led to delays in physical and mental development of the offspring resembling hypothyroidism. There are indications that

organochlorine compounds may affect neonatal neurological development, possibly by affecting thyroid hormone status.

**Wildlife effects**

Many wildlife species may be exposed to biologically active concentrations of endocrine disrupting chemicals. There is strong evidence obtained from laboratory studies showing the potential of several environmental chemicals to cause endocrine disruption at environmentally realistic exposure levels. In wildlife populations, associations have been reported between reproductive and developmental effects and endocrine disrupting chemicals. Effects have been observed in mammals, birds, reptiles, fish and molluscs from Europe, North America and other continents, in particular of the aquatic environment. The observed abnormalities vary from subtle changes to permanent alterations, including disturbed sex differentiation with feminized or masculinized sex organs, changed sexual behaviour, and altered immune function. For most reported effects in wildlife, however, the evidence for a causal link with endocrine disruption is weak or non-existing. Crucial in establishing causal evidence for chemical-induced wildlife effects appeared semi-field or laboratory studies using the wildlife species of concern. Although most observed effects currently reported concern heavily polluted areas, there is a potential global problem. This is exemplified by the widespread occurrence of imposex in marine snails and the recent findings of high levels of persistent potential endocrine disrupting chemicals in several marine mammalian species inhabiting oceanic waters. Impaired reproduction and development causally linked to endocrine disrupting chemicals are well documented in a number of species and have caused local or regional population changes. These include:

- masculinization (imposex) in female marine snails by tributyltin, a biocide used in anti-fouling paints, is probably the clearest case of endocrine disruption caused by an environmental chemical. The dogwhelk is particularly sensitive and imposex has resulted in decline or extinction of local populations worldwide, including coastal areas all over Europe and the open North Sea.
- DDE-induced egg-shell thinning in birds is probably the best example of reproductive impairment that caused severe population declines in a number of raptor species in Europe and North America. Developmental exposure to the DDT complex has been firmly linked to the induction of ovotestis in male Western gulls.
- endocrine disrupting chemicals have adversely affected a variety of fish species. In the vicinity of certain sources (e.g. effluents of water treatment plants) and in the most contaminated areas is this exposure causally linked with effects on reproductive organs which could have implications for fish populations. However, there is also a more widespread occurrence of endocrine disruption in fish in the United Kingdom, where oestrogenic effects have been demonstrated in freshwater systems, in estuaries and in coastal areas.
- in mammals, the best evidence comes from the field studies on Baltic grey and ringed seals, and from the semi-field studies on Wadden Sea harbour seals, where both reproduction and immune functions have been impaired by PCBs in the food chain. Reproduction effects resulted in population declines, whereas suppression of immune function have likely contributed to the mass mortalities due to morbillivirus infections.
- distorted sex organ development and function in alligators has been related to a major pesticide spill into a lake in Florida, U.S.A. The observed oestrogenic/anti-androgenic effects in this reptile have been causally linked in experimental studies with alligator eggs to the DDT complex.

**Ecological risk assessment and toxicological test guidelines**

Ecological risk assessment is intended to evaluate risks on the structure and functioning of ecosystems. The strategy for ecotoxicity assessment must focus on relevant endpoints for the detection of population-community effects. The analysis of current protocols for ecological risk assessment indicates a concern on the capability of low tier levels to detect the ecological risk of endocrine disrupters because of problems related to the suitability of the test species and the extrapolation from acute lethality to long-term effects.

**Toxicological test guidelines and testing strategies**

Based on the present review of ecotoxicology and toxicology regulatory test guidelines, the CSTEE comes to the following conclusions:

- present regulatory toxicology test guidelines, in particular the guidelines for ecotoxicity testing, cannot detect all endocrine disrupting effects. Therefore, current test guidelines have to be enhanced or new guidelines developed. In this process, international co-operation (EU, OECD, EMSG) is essential to avoid duplication.
- reliance on *in vitro* assays for predicting *in vivo* endocrine disrupter effects may generate false-negative as well as false-positive results. Thus, the development of *in vitro* prescreening test methods is not recommended. In stead, major emphasis should be put on *in vivo* assays.
- the current enhancement by the OECD of the existing 407 repeated oral toxicity test in rodents and the existing OECD 416 reproduction toxicity test has high priority support.

**Recommendations**

The CSTEE makes the following recommendations:

- to further evaluate the human health effects which have been associated with endocrine disrupters and to identify the underlying causes. In this, special attention should be given to exceptional high chemical exposures and to the health consequences of phytoestrogens in human food.
- to conduct further field and semi-field studies to establish cause-and-effect relationships. In this respect it is important to establish baseline data in non-exposed reference populations.
- to encourage the establishment of co-ordinated biomonitoring programmes to assess the full environmental significance of endocrine disruption for wildlife, including studies on effects in terrestrial systems and in amphibian and reptile populations in Europe.
- to establish the long-term consequences at the population and community level of the observed effects in reproductive organ structure and function (such as vitellogenin induction, precocious female maturation, intersexuality, ovotestis formation, and altered sexual behaviour), thyroid status and immune system.
- to establish the capability of laboratory long-term tests to detect ecologically relevant effects related to endocrine disruption.
- to determine which margins of safety and uncertainty factors must be considered, in the case of endocrine disrupters, for the extrapolation of ecotoxicological thresholds from acute and chronic toxicity studies.
- to consider when species-focused assessments may be required and to develop decision making schemes and risks assessment procedures for critical wildlife species which require a complementary level of protection, such as endangered species.
- for fish, it is recommended to enhance the early life-stage test and to further development the partial life-cycle test. Besides these screening assays, the confirmatory full life-cycle test needs development.
- for birds, the present one-generation test guideline should be enhanced.

- for invertebrates, the existing tests should be implemented by the inclusion of appropriate endpoints which cover full life-cycle effects related to endocrine disruption. Testing strategies should include several invertebrate species.
- for new chemicals produced in less than 1 tonne/year it should be considered whether there is a need to additional toxicity testing for reproductive toxicity (currently only if >1 t/year) or fertility (currently only if >10 t/year). In that case, new criteria for decision should be developed that are also based on structure-activity relationships.
- for new and existing chemicals the requirements for data on reproductive effects should be harmonised and the technical guidance document updated with focus on testing strategy for endocrine disruption.

# 1. Introduction

## 1.1 Background

There is growing concern on possible harmful consequences of exposure to xenobiotic compounds that are capable of modulating or disrupting the endocrine system. This concern for endocrine disrupting chemicals (EDCs) is directed at both wildlife and humans (Colborn et al., 1993; Sharpe and Skakkebaek, 1993). Several expert working groups (Harrison et al., 1995; Toppari et al., 1995; Weybridge, 1996 ) have concluded that there is increasing evidence of adverse effects in human and wildlife reproductive health, and have discussed the hypothesis that chemicals in the environment have caused these endocrine mediated adverse effects. This has led a series of stakeholders to consider the topic of endocrine disruption as of sufficient interest/concern to justify action.

Among the stakeholders mentioned are worthy of mention the European Commission, the European Parliament, the US Environmental Protection Agency, OECD, the IPCS, the Commission of the OSPAR Convention, the European Environment Agency, NGOs and the Chemical Industry.

The media and consequently the public at large have therefore developed an interest on the subject. Several parliamentary questions were asked on it and the European Parliament specifically requested the Commission to foster initiatives in the areas of research, legislation and public information.

Concerning in particular the involvement of the European Commission, the Commission Communication of 30 April 1997 on Consumer Health and Food Safety and the Commission Decision of 23 July 1997 setting up Scientific Committees in the field of consumer health and food safety have given the Scientific Committees currently being managed under DG XXIV Consumer Policy and Consumer Health Protection, amongst others, the task to draw the attention of the Commission to potential or emerging hazards relating to consumer health.

Both the previous Scientific Advisory Committee for the examination of the Toxicity and Ecotoxicity of chemical compounds and the Scientific Committee that replaced it, the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE), have advised that the issue of endocrine disrupting chemicals (EDCs) be given a high priority by the Commission within the framework of its actions to protect consumer health.

The Commission took this advice seriously and allowed for the setting up of a Working Group on Endocrine Disrupters under the CSTEE. The working group set out to produce the report below whose focus is clearly established in its table of contents. The working group believes that, in terms of endocrine disrupting effects, the main reasons for concern have to do with the effects on wildlife and the environment and the European situation is evaluated correspondingly. Furthermore test methodology also deserves a more proper focus and this is reflected in the current opinion.

On the policy side the European Commission services are in the process of finalising a Community Strategy for Endocrine Disrupters which focuses on mam-made chemicals and addresses the key requirements for further research, international co-operation, communication to the public and appropriate policy action. Independent scientific advice is acknowledged as an important element of the Strategy.

### 1.2 Mandate

Given the above, the CSTEE's role is to advice the Commission on the current state of the art regarding knowledge of EDCs. This applies as much to effects on human health as on effects on wildlife and the environment.

Given the uncertainties characterising the current knowledge on the subject, the CSTEE should also inform the Commission about the correct strategies to follow regarding test methodologies. These should in principle be targeted to those areas of knowledge more in need of research.

The remit of the CSTEE is to be understood as one of the elements the Commission needs in its developing of an appropriate policy line on EDCs. In this respect the CSTEE acknowledges the role played by other international bodies, in particular the OECD, as the focal point under which the most significant developments on EDCs knowledge are taking place. A reflection of this acknowledgement is the participation of CSTEE members in those activities, to which they are regularly invited.

The current opinion on EDCs is therefore in line with the above-mentioned needs. The emphasis of this paper is a review of the existing literature and scientific opinion on the evidence for chemically-induced endocrine disruption, in particular as it relates to the European wildlife, on EU testing strategy, on ecological risk assessment and on toxicological test guidelines. In addition, gaps in knowledge are identified and recommendations for future research made. The CSTEE hopes that this paper will be of assistance to the European Commission in its involvement on the topic and help it sort out some of the problems it faces, particularly as the resources are scarce when compared with what needs to be done in order to clarify the situation.

Finally, by its very nature, knowledge on the subject of EDCs is likely to change significantly, as evidence becomes available. This should be typically the case during perhaps the next four to five years if one draws on the activities foreseen for the short and medium term future. This means that the involvement of the CSTEE should continue on a sort of 'on call' basis wherever there will be a need to either answer specific questions, evaluate the new knowledge or even put forward proposals in order to keep up with technical progress.

### 1.3 Definition of endocrine disruption

The working group agreed upon the definition of the IPCS Steering Group that met at the joint IPCS/OECD Scoping Meeting on Endocrine Disrupters, 16-18 March 1998 in Washington, DC:
**An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.**
**A potential endocrine disrupter is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations.**

Alteration of endocrine function caused by an endocrine disrupter may be through interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour. Industrial chemicals, consumer chemicals and chemicals in the environment can be endocrine disrupters that mimic, enhance (an agonist) or inhibit (an antagonist) the action of hormones. Dose, body burden, timing, frequency and duration of exposure at critical periods of life

are important considerations for assessing adverse effects of an endocrine disrupter. In this paper, stress-related effects on health that are mediated by the pituitary-adrenal axis are not discussed.

### 1.4 Mechanisms of endocrine disruption

The biological actions of hormones synthesised within an organism, such as oestrogen, progesterone, testosterone and thyroxine are mediated by high-affinity receptor proteins located within target cells. The interaction of a hormone with its receptor initiates a cascade of events that lead to the myriad of effects associated with the particular hormone. Also exogenous chemicals may bind to a receptor, e.g. the oestrogen receptor and mimic or block the actions of its natural hormone. Such compounds include some natural products such as coumestrol and genistein, pharmaceuticals such as diethylstilbestrol, 17α–ethinyloestradiol and tamoxifen, and industrial chemicals such as DDT, bisphenol A and nonylphenol (ECETOC, 1996; Schäfer et al., 1996; DFG, 1996; European Commission 1996). Compounds which have been shown to alter oestrogen biosynthesis are cyanoketone, ketoconazole and the fungicide fenarimol (Hirsch et al., 1987). Methoxychlor, chlordecone (kepone), DDT, some PCB and alkylphenols can disrupt oestrogen receptor function (White et al., 1994, Mueller and Kim, 1978). Metabolites of the fungicide vinclozolin and the DDT metabolite *p,p'*-DDE have been found to bind to the androgen receptor and block testosterone-induced cellular responses *in vitro*. *o,p'*-DDT and chlordecone can inhibit ligand binding to the oestrogen and progesterone receptors (Laws et al., 1995). Nonylphenol and the other synthetic chemicals inhibit binding to the oestrogen, progesterone and androgen receptors (Schäfer et al., 1996). Environmental agents have also shown to affect the thyroid. For example, hydroxylated metabolites of PCBs, as competitive inhibitors of thyroxine binding to transthyretin, can alter thyroid hormone levels and interfere with hormone transport.

The steroidal sex hormones and their receptors are phylogenetically old and may be found in a range of vertebrate and evertebrate species. Their regulatory role in developmental processes such as sex determination and differentiation is of particular interest with regard to endocrine disruption. In mammals, the differentiation of the conceptus into a male phenotype is regulated by testicular hormones including testosterone. Consequently, the androgen receptor antagonists *p,p'*-DDE and vinclozolin may demasculinize male rat pups following exposure of the pregnant dam (Kelce et al., 1995; 1997). In birds, the differentiation into a female phenotype is regulated by oestrogen. Consequently, injection of an oestrogen synthesis (aromatase) inhibitor into the fertilized egg may result in phenotypically sex-reversed females with bilateral testicles, sperm production and a male copulatory behaviour (Elbrecht and Smith, 1992). Feminised male birds with an ovotestis (a gonad containing both ovarial and testicular tissue) and/or reduced male copulatory behaviour have been induced by *o,p'*-DDT and 17α–ethinyloestradiol (Adkins, 1975; Schumacher et al., 1989; Berg et al., 1998).

The genetic sex in mammals and birds is defined by genes located on the sex chromosomes. In lower vertebrates and evertebrates, the mechanisms of sex determination and differentiation are less characterized but may be more flexible than in mammals and birds and influenced by environmental factors such as temperature and xenogenous substances with hormonal activity. Experimentally disturbed sex differentiation has also been reported in reptiles, amphibia, fish and evertebrates such as marine gastropods. There is evidence that malformed sex organs occur in wild populations of birds, reptiles, fish and marine gastropods (see chapter 3). The mechanisms of pollutant-induced reproductive toxicity observed in wild mammalian species generally remain unclear but could also involve endocrine disruption.

## 2. EDCs and human health

This overview briefly summarises the available evidence for human health effects of EDC on male reproductive tract, on certain types of cancer, on fertility, on the thyroid, and on the central nervous system.

### 2.1 Effects on the male reproductive tract

#### 2.1.1 Decreased sperm quality

Though controversy persists about the allegation that human sperm production has been declining world-wide during the past 40 to 50 years, a hypothesis that in utero exposure to environmental oestrogens might be responsible has been proposed (Sharpe and Skakkebaek, 1993).

A meta-analysis of 61 studies that included 14 947 men found a substantial decrease in sperm concentration from 113 x $10^6$/ml in 1938 to 66 x $10^6$/ml in 1990 and in semen volume from 3.40 x $10^{-3}$/L in 1938 to 2.75 x $10^{-3}$/L in 1990 (Carlsen et al., 1992). Of the 61 studies in the Carlsen et al. study, only 13 are from before 1970, and the results from these studies define the upper end of the regression curve. The study by Carlsen et al. (1992) can be questioned since it suffers from various sources of bias and confounding (Mees et al., 1997). In a reanalysis of the 48 studies published since 1970, sperm counts actually increased between 1970 and 1990 (Golden et al., 1998). Another reanalysis of Carlsen's data showed no change in the sperm quantity (Golden et al., 1998, Jensen et al. 1995). There are several additional studies of sperm quality which have a relatively good design (Auger et al., 1995; Adamapoulus et al., 1996; Paulsen et al., 1996, Fisch et al., 1996). two of these studies observed a decline in sperm concentration, one found no change and one detected an increase. All four studies observed an unchanged seminal volume or even a slight increase.

Decreased sperm count (115 x $10^6$/ml in 87 controls vs. 91 x $10^6$/ml in 134 DES-exposed) men and abnormal Eliasson scores in 18% of 134 DES-exposed men compared with 8% in 87 placebo-exposed controls have been reported (Golden et al., 1998). This study was conducted on a cohort of men exposed *in utero* to DES according to the dosing protocol in use at the University of Chicago, where mean total maternal DES administered amounts were, on average, 11.603 g. In contrast, in a cohort of men from the Mayo Clinic exposed in utero to DES at total mean maternal DES administered amounts of approximately 1.4 g, there were no differences between 100 DES-exposed men and unexposed men in sperm count or motility (Golden et al., 1998).

The studies mentioned above may be confounded by the following:

- The validity of comparing results obtained from different populations of men from different geographic areas and different times.
- The lack of control for abstinence time before provision of the semen sample.
- Difference in the laboratory quality and counting methodology.

Additional carefully designed and conducted epidemiological studies are required to substantiate and clarify the cause of any reduction of sperm counts.

A sperm count decline is not necessarily associated with infertility. Seibert (1997) reports that although in recent years an increase in oligospermia in men (< 20 x $10^6$ sperm/ml) is evident, the number of involuntarily childless couples did not increase. In 1994 (Olsen, 1994) it was reported that there had not been an increased incidence of infertile pairs during the last 10 years in the

industrial countries. But, it should be noted that methodological difficulties exist in investigating male infertility in descriptive as well as *ad hoc* epidemiological studies.

In utero exposure to DES caused a decrease in sperm count, but no effect on fertility was shown (Golden et al., 1998). It is still to be decided whether a moderate sperm count decrease is an adverse effect.

### 2.1.2 Cryptorchidism

An increase in the prevalence of cryptorchidism was reported in some investigations (Jensen et al., 1995, Golden et al., 1998). Because of differences in examination techniques and in the age groups of boys examined, the results of the various studies are difficult to compare. Only a few studies include different ethnic groups. The incidence among American blacks was reportedly only one-third of that among whites (see Golden et al., 1998), although another study found no substantial difference between whites and blacks (Berkowitz et al., 1995). Very few studies examine temporal changes in prevalence. Therefore, additional epidemiological studies are needed to confirm the data.

### 2.1.3 Hypospadia

Several reports have indicated temporal increase in the prevalence of hypospadias (Jensen et al., 1995). The prevalence of hypospadias varies considerably in the different studies, from 0.37 to 41 per 10000 infants, and interpolation comparisons are difficult because of possible differences in ascertainment and inclusion of less severe cases. The increasing incidence of hypospadias was reported primarily in England and Wales, Hungary, Sweden, Norway, Denmark, Finland, Spain, New Zealand, Australia, and Czechoslovakia. The prevalence may be higher in whites than in blacks in the United States. The prevalence in Finland is considerably lower (5 per 10 000) than in other Scandinavian countries (Denmark: 14 per 10 000). However, more epidemiological studies should be undertaken in order to address this issue.

## 2.2 Testicular cancer

The incidence of testicular cancer in men has increased significantly during the last 30 years (Forman and Møller, 1994). The tumors are primarily germ cell in origin. Toppari et al. (1995) have estimated that cancer incidence in men under the age of 50 has increased approximately 2% to 4% per annum since the 1960s in Great Britain, the Nordic and Baltic countries, Australia, New Zealand and the United States. There are marked differences in incidence levels between countries. Testicular cancer is the most common malignancy among men age 25 to 34, with age-specific incidence as high as approximately 25 per $10^5$ in Denmark (Adami et al., 1994). Interestingly, the corresponding incidence in Finland is about 5 per $10^5$. The reason for this difference is not known. Most of the tumors occurring in young men are germ cell in origin.

It has not yet been possible to conclude whether the apparent increase in this type of cancer in many countries is indeed due to hormonally active substances, to changed life-style conditions or to other causes. The Danish environmental authorities expressed doubt about a relationship of testicular cancer to hormonally active substances in their report of 1995. They substantiated this with the remark that, although in the USA an increase in testicular cancer can be recognised within the white population, this does not apply to the population of Asian or African descent (Toppari et al., 1995). Further thorough epidemiological studies as to the causes for the increases in incidence of testicular cancer are essential.

## 2.3 Prostate cancer

Carcinoma of the prostate is the second leading cause of cancer deaths in males in the United States. Death due to prostate cancer has increased by 17% over the past 30 years despite improved diagnosis. There are racial differences in susceptibility, the prevalence being rare in Orientals, 20 to 30 times higher in Caucasians, and even higher in African-American males (40% higher than whites) (Crisp et al., 1998).

A meta-analysis found positive associations between prostate cancer and farming from all studies published between 1983 and 1994, but no association was found from studies only reporting a standard mortality ratio (Keller-Byrne et al., 1997).

Little is known about the causes of prostatic cancer, but age, genetics, endocrine status, diet and environmental risk factors have been proposed. More epidemiological research is required to elucidate whether there is a causal correlation between prostate cancer and endocrine disrupter exposure.

**2.4 Breast cancer**

Cancer of the breast is the most frequent tumour in females in the world. The relative frequency varies five-fold between countries, the highest risk for breast cancer are found in western Europe and in North America. There is almost a two-fold difference between the highest and lowest incidence rates in Europe (Switzerland vs. Spain). There has has been a steady increase in breast cancer incidence rates over the last decades everywhere in Europe. Estimated mean changes per five-year period in age specific rates (30-74 years) over the period 1973-87 are in the order of 5-25 per cent (Coleman et al., 1993). Several factors have been identified as potentially responsible for increasing the risk, but their mechanism of action is unclear. Reproductive history obviously plays a role since early menarche, late first pregnancy, low parity and late menopause are all associated with an increased risk of breast cancer. The increase in risk has been suggested to be related to exposure to oestrogenic chemicals, however, no specific exposures have been identified. Oestrogenic influences may be important since oophorectomy has a protective effect on breast cancer development. There is still limited evidence for oral long-term, contraceptive use being a risk factor.

Several case-control studies published in the last two decades raised the issue that women exposed to oestrogen-like chemicals such as DDT and certain PCB congeners may have higher incidences of breast cancer than non-exposed women (Wasserman et al., 1976; Falck et al., 1992; Wolff et al., 1993; Dewailly et al., 1994). In a recent study including 268 cases from a cohort of 7712 women the plasma concentration of dieldrin was positively associated with an increased risk of breast cancer (adjusted odds ratio 2.05 (95% CI 1.17-3.57) (Høyer et al., 1998). However, the dieldrin concentrations were orders of magnitude lower than those able to produce a (weak) oestrogenic response *in vitro*. In the same study there was no association to the total PCB, total DDT, or HCB. Other studies have failed to identify associations between organochlorine exposure and breast cancer (Krieger et al., 1994; Van't Veer et al., 1997; Hunter et al., 1997). Two of these latter studies (Van 't Veer et al., 1997; Hunter et al., 1997) reported lower adipose levels of the DDT metabolite DDE in cancer patients than among controls. A systematic review of the epidemiological findings regarding the association between organochlorines and breast cancer found these to be inconclusive (Ahlborg et al., 1995). Available data support a possible weak association between diethylstilboestrol exposure during pregnancy and later development of breast cancer (Hadjimichael et al., 1984; Vessey et al., 1983; Greenberg et al., 1984; Colton and Greenberg, 1993).

There is no evidence from experimental studies that DDT, DDE or PCB produce increased incidences of mammary tumours in rodents. However, administration of diethylstilboestrol,

genistein and *o,p'*-DDT has resulted in enhanced epithelial cell proliferation and differentiation of abdominal mammary glands in female Sprague-Dawley rats (Brown and Lamartiniere, 1995). TCDD, which may act as an antioestrogen (Safe et al., 1991), reduced the number of spontaneous tumours in the mammary gland in a 2-year carcinogenicity bioassay (Kociba et al., 1978).

**2.5 Endometriosis**

Endometriosis is characterised by aberrant growth of endometrial cells outside the uterus and the ensuing dysmenorrhoea due to sloughing of the oestrogen-induced proliferation tissue and the internal bleeding that follows. Prevalence estimates range widely with an average of 10 per cent and affects 5 million women in the United States (Olive and Barrie Schwartz, 1993; Holloway, 1994). The etiology of this disease is unknown, but several hypotheses have been put forward, including exposure to oestrogen-like chemicals. Also, immune mechanisms may be involved in the disease process (Hill, 1992). Some studies report increased risks of endometriosis in women formerly taking oral contraceptives, whereas there was a decreased risk in women currently taking such medication (Vessey et al., 1993). Also, there is evidence of an increased frequency of endometriosis in female offspring exposed *in utero* to diethylstilboestrol (Stillman and Miller, 1984; Berger and Alper, 1986).

An association between women with endometriosis and high levels of PCB has been reported (Gerhard and Runnebaum, 1992). On the other hand, no significant correlations between endometriosis and serum levels of PCDD, PCDF or PCB were found in a small study where disease status was determined by laparoscopy (Boyd et al., 1995).

In an investigation of dietary TCDD intake in rhesus monkeys, a dose-response between dioxin exposure and the incidence and severity of endometriosis was observed (Rier et al., 1993). In contrast, dietary PCB exposure did not alter endometrial lesions in rhesus monkeys, even at doses that were 10 times higher than the Rier et al. (1993) study based on TCDD-receptor binding (Arnold et al., 1996). In a recent study with rats and mice, TCDD was found to promote endometriosis in both species, although the magnitude of the effects was greater in mice than in rats (Cummings et al., 1996). At the same time, ovarian weights were reduced in rats but not in mice, and both species had developed thymic atrophy after TCDD treatment.

More epidemiological studies addressing possible links between exposure to hormonally active chemicals and endometriosis are clearly warranted.

**2.6 Effects on fertility including sex ratio**

Infertility may be defined as a couple's failure to conceive after a period of 1 year of unprotected intercourse, and may affect between 10 to 15 percent of couples. It is estimated that up to 50% of all fertility problems may be related to male reproductive function (Swerdloff, 1985). There are well documented cases of severely affected male reproductive function from occupational exposures such as dibromochloropropane, lead and kepone causing direct or indirect testicular toxicity (Schrag and Dixon, 1985). Although one study found three times more genital malformations in men with *in utero* exposure to diethylstilboestrol than controls, there was no significant difference in fertility (Wilcox et al., 1995). Increases in blood levels of *p,p'*-DDT, DDD, DDE, lindane, tetra- and pentachlorobiphenyls have been found more frequently in individuals with sperm counts below 20 million/ml than in those with sperm counts above 20 million/ml (Pines et al., 1987). On the other hand, in 36 transformer repair workers with a median blood PCB level of 12 ppb and 56 comparison workers with a median PCB level of 6 ppb, no differences in sperm count were found (Emmett et

al., 1988). However, the PCB pattern in people exposed occupationally may be quite different from the pattern following dietary exposure.

Conflicting results have come from studies examining fertility and pregnancy outcomes of women exposed *in utero* to diethylstilboestrol. Whereas some studies report no differences in fertility rates (Barnes et al., 1980; Cousins et al., 1980), another investigation found that 18 percent of diethylstilboestrol-exposed women achieved pregnancy compared to 33 percent in unexposed (Bibbo and Gill, 1977). However, it is well established that diethylstilboestrol exposure *in utero* leads to greater incidence of unfavourable pregnancy outcomes such as miscarriage, ectopic pregnancy, stillbirth and premature birth (Barnes et al., 1980). Daily oral intake of approximately 20-50 µg ethinyloestradiol plus varying amounts of progesterone are required to cause infertility by oral contraceptives (Isselbacher et al., 1994).

Male to female sex ratio has been used as an indicator of human fertility. After the Seveso accident with exposure to dioxin, there was an excess of females born in the period April 1977 to December 1984 (Mocarelli et al., 1996). This ratio declined in the years from 1985 to 1994 and was thereafter no longer significant. There have also been reports of declining proportion of male new-borns during the last decades from Denmark (Møller, 1996), The Netherlands (Van der Pal-de Bruin et al., 1997), England and Wales (Dickinson and Parker, 1996), as well as from Canada (Allan et al., 1997). This decline in the sex ratio remains unexplained, but has been speculated to be related to exposure to xeno-oestrogenic chemicals.

### 2.7 Effects on the thyroid

Thyroid hormones play a key role in the maintenance of body homeostasis. Altered thyroid status may lead to changes in basal metabolic rate, lipid metabolism as well as cardiovascular, gastrointestinal and muscle function. Thyroid hormones are especially important during growth and development such as the maturation of the brain. A number of environmental agents can alter thyroid hormone levels in humans and animals. Hypothyroidism in rodents has been observed after exposure to PCB, TCDD, and chlorinated pesticides (Crisp et al., 1998).

Perturbations of thyroid hormone balance leading to prolonged compensatory secretion of thyroid stimulating hormone may lead to increased incidences of thyroid follicular cell tumours in rats (Capen, 1992). It should be recognised that there are substantial differences in thyroid physiology between rodents and humans. The weight of evidence suggests that rodents are more sensitive than human subjects to thyroid tumour induction due to hormonal imbalances resulting in elevated thyroid stimulating hormone levels (IARC, 1998).

Thyroid cancer is an uncommon tumour in the age range 30-74 years, it usually represent less than 2% of all malignant neoplasms (Coleman et al., 1993). Incidence rates are two or three times higher in females than in males, although this sex difference declines after middle age. Among European populations, the highest incidence rates in both sexes have been observed in Switzerland and in the Nordic countries. Incidence has risen in the Nordic countries and East Germany during the last decades, changed very little in West Germany, whereas in Switzerland there has been a sharp decline.

Accidental exposure to PCB and PCDF by pregnant women in Taiwan led to a number of delays in physical and mental development of their offspring, similar to those associated with hypothyroidism (Hsu et al., 1985). Higher PCDD, PCDF and PCB levels in human milk has been found to correlate with lower plasma levels of maternal thyroid hormones and with higher plasma levels of thyroid stimulating hormone in infants shortly after birth (Koopman-Esseboom et al., 1994). Enhanced

levels of these environmental contaminants in breast milk have been related to reduced neonatal neurological function (Huisman et al., 1995).

Hydroxylated PCBs have been found to be potent, competitive inhibitors of thyroxine binding to the human thyroid hormone transport protein transthyretin (Lans et al., 1993).

**2.8 CNS and neuroendocrine effects**

Direct effects on endocrine glands such as the thyroid may alter the hormonal homeostasis which in turn can affect the nervous system, resulting in neurotoxicity. Direct effects on the hypothalamic-pituitary axis may result in altered sexual differentiation in the brain, or affect sexual dimorphic endpoints.

Children born to women exposed to PCB/PCDF-contaminated fish oil or rice oil have been reported to show delayed mental development with lower IQ scores, cognitive dysfunction, poorer visual recognition memory, and behavioural difficulties. A Dutch study observed a significant delay of psychomotor development in children pre- and postnatally exposed to PCBs, PCDDs, and PCDFs (Koopman-Esseboom et al., 1996).

Developmental neurotoxicity involving cognitive and neurobehavioral disturbances have been implicated following perinatal exposure to environmental pollutants. Mainly PCBs have been implicated (Jacobson and Jacobson, 1996). There are several studies which show that PCBs produce a wide spectrum of neurochemical and neuroendocrine effects in animals. Ortho-substituted PCB congeners affect brain neurochemistry, while non-ortho-substituted PCBs having dioxin-like activity may have little or no activity in the nervous system (Tilson and Kodavanti 1997).

Polybrominated biphenyl ethers (PBDEs) have been identified in human plasma and milk samples and a preliminary paper by Eriksson et al. (1998) reported that neonatal exposure to some congeners may cause disturbed spontaneous behaviour similar to that described for PCBs.

At present it is not known whether the reported developmental neurotoxicities related to PCB/PCDF/PCDD/PBDE exposures are caused by endocrine mechanisms.

**2.9 Exposure considerations**

Numerous investigations on the concentrations of EDC in human food and tissues and the relative potency of these chemicals in vitro as compared to oestradiol are available (Gaido et al., 1997; Sonnenschein et al., 1995; Soto et al., 1995; Wang et al., 1996; Zava and Duwe, 1997). For assessing the relative risk of the endocrine disrupters, human exposure to these compounds expressed as their concentration in serum was related to the oestrogenic activity determined in *in vitro* tests as the effective concentration of the compound showing 50 % or 100 % of the maximum oestrogen activity (see Tables 1-3). However it should be realized that the binding to sex globulin binding protein may be relatively greater for the endogenous hormones. Data on the oestrogen activities are taken from experiments with the following test systems: competitive binding to oestrogen receptor of MCF-7 cells, proliferation of MCF-7 human breast cancer cells (E-SCREEN) or expression of a reporter gene in the yeast oestrogen system (YES). The results show that the relative potencies of *o,p'*-DDE, PCBs, nonylphenol, bisphenol and dieldrin in *in vitro* systems as compared to that of oestradiol are several orders of magnitude smaller. The phytoestrogen genistein however with both an higher oestrogen activity and higher serum concentrations than pesticides has a relative potency which can even exceed that of oestradiol in the case of diets rich in soy. It is well documented that the concentration of genistein varies over a wide range in individuals consuming

diets with low or high soy content (see Tables 1 and 2). This leads to a wide range of the relative oestrogenic potency of genistein.

The intake of phytoestrogens from food varies widely among different populations (British < 1 mg/d in Asian countries up to 100 mg/d), depending on their dietary habits (Cassidy, 1998). In any case, it can be stated that the major human intake of endocrine disrupters are naturally-occurring oestrogens found in foods (Safe, 1995). This exposure is several orders of magnitude higher than the exposure to pesticide EDCs. More data are needed for a more accurate assessment of the internal exposure to phytoestrogens and to EDC.

Safe (1995) calculated the daily human intake of oestrogen and antioestrogenic equivalents, based on potencies relative to 17β−oestradiol. It was shown that a woman taking a birth control pill ingests about 16,675 μg equivalents per day, postmenopausal oestrogen therapy amounts to 3,350 μg, ingestion of oestrogen flavonoids in food represents 102 μg, whereas daily ingestion of environmental organochlorine oestrogens was calculated to be 0.0000025 μg. In case of naturally occurring chemicals such as the phyto-oestrogens (isoflavones) an effect has been shown after intake of soya protein (60g/day) in women. These substances have activities ranging from 1/500 to 1/1000 of 17β−oestradiol.

The critical element for risk assessment is exposure assessment as normally insufficient information is available. This is especially true in the case of hormonally active chemical exposures. The dose response or dose effect relationship for some of the compounds to be considered is not well elucidated in man. It must be realised that for some hormonally active compounds the most sensitive target may not have been identified, which would complicate the risk assessment.

**Table 1: Relative Binding of Endocrine Modulators to the Oestrogen Receptor in MCF-7 cells**

| Compound | $IC_{50}$ A) [Mol/l] | Concentration in human serum B) | Relative Potency | Reference |
|---|---|---|---|---|
| Oestradiol | $2 \times 10^{-9}$ | 187 ng/l (0.69 nM) | 1 | (A) Soto et al., 1995; Wang et al., 1996<br>(B) Lu et al., 1996 |
| 4-Nonyl-phenol | $7 \times 10^{-6}$ | < 1ng/l (4.5 pM) | $2 \times 10^{-6}$ | (A) Soto et al., 1995<br>(B) Müller, 1997 |
| *o,p'*-DDT | $5 \times 10^{-4}$ | 0.06 µg/l (0.17 nM) | $10^{-6}$ | (A) Soto et al., 1995<br>(B) Anderson et al., 1998 |
| Genistein | $5 \times 10^{-7}$ | 0.006 µM [a] - 5 µM [b] | 0.03 - 29 | (A) Wang et al., 1996<br>(B) Adlercreutz et al., 1993; Xu et al., 1995 |
| Oestradiol | $2 \times 10^{-10}$ | 187 ng/l (0.69 nM) | 1 | (A) Zava and Duwe, 1997<br>(B) Lu et al., 1996 |
| Genistein | $2 \times 10^{-7}$ | 0.006 µM [a] - 5 µM [b] | $9 \times 10^{-3}$ - 7 | (A) Zava and Duwe, 1997<br>(B) Adlercreutz et al., 1993; Xu et al., 1995 |

A) $IC_{50}$: concentration leading to 50 % inhibition of competitive binding of $^{3}H$-oestradiol to the oestrogen receptor

B)Relative Potency: ratio of concentration in human serum to effective concentration, normalized to that of oestradiol

a) serum concentration of Finnish people consuming food with a low genistein content

b) serum concentration of a study population consuming a soy diet rich in genistein

**Table 2: Oestrogenic Potency of Endocrine Modulators in the E-SCREEN test**

| Compound | Effective concentration [Mol/l] A) | Concentration in human serum | Relative Potency B) | Reference |
|---|---|---|---|---|
| Oestradiol | $3 \times 10^{-11}$ | 187 ng/l (0.69 nM) | 1 | (A) Sonnenschein et al., 1995; Soto et al., 1995<br>(B) Lu et al., 1996 |
| 4-Nonyl-phenol | $1 \times 10^{-6}$ | < 1ng/l (4.5 pM) | $2 \times 10^{-7}$ | (A) Sonnenschein et al., 1995, Soto et al., 1995<br>(B) Müller, 1997 |
| Bisphenol A | $1 \times 10^{-6}$ | < 1ng/l (4.4 pM) | $2 \times 10^{-7}$ | (A) Sonnenschein et al., 1995<br>(B) FDA, 1995 |
| PCBs | $1 \times 10^{-6}$ - $1 \times 10^{-5}$ | 6.7 µg/l (26 nM) | $1 \times 10^{-3}$ - $1 \times 10^{-4}$ | (A) Soto et al., 1995<br>(B) Wolff et al., 1993 |
| Dieldrin | $1 \times 10^{-5}$ | 0,2 ng/l (0.52 pM) | $2 \times 10^{-9}$ | (A) Soto et al., 1995<br>(B) Anderson et al., 1998 |
| *o,p'*-DDT | $1 \times 10^{-5}$ | 0.06 µg/l (0.17 nM) | $7 \times 10^{-7}$ | (A) Soto et al., 1995<br>(B) Anderson et al., 1998 |
| *p,p'*-DDT | $1 \times 10^{-5}$ | 0.2 µg/l (0.56 nM) | $2 \times 10^{-6}$ | (A) Soto et al., 1995<br>(B) Göen and Angerer, 1997 |
| Oestradiol | $1 \times 10^{-9}$ | 187 ng/l (0.69 nM) | 1 | (A) Zava and Duwe, 1997<br>(B) Lu et al., 1996 |
| Genistein | $1 \times 10^{-7}$ | 0.006 µM [a] - 5 µM [b] | 0.09 - 72 | (A) Zava and Duwe, 1997<br>(B) Adlercreutz et al., 1993; Xu et al., 1995 |

A) Effective concentration: the lowest reported concentration needed for maximal yield of MCF-7 cell proliferation; the maximal cell yield may be different for each tested compound

B) Relative Potency: ratio of concentration in human serum to effective concentration, normalized to that of oestradiol

a) serum concentration of Finnish people consuming food with a low genistein content

b) serum concentration of a study population consuming a soy diet rich in genistein

**Table 3: Estimated Relative Potency of Endocrine Modulators in the Yeast-Based Oestrogen Receptor Assay**

| Compound | Effective concentration [Mol/l] A) | Concentration in human serum | Relative Potency B) | Reference |
|---|---|---|---|---|
| Oestradiol | $2.25 \times 10^{-10}$ | 187 ng/l (0.69 nM) | 1 | (A) Gaido et al., 1997<br>(B) Lu et al., 1996 |
| Testosterone | $5.09 \times 10^{-5}$ | 6 µg/l (20.6 nM) | $1.3 \times 10^{-4}$ | (A) Gaido et al., 1997<br>(B) Jungermann and Möhler, 1980 |
| *o,p'*-DDT | $1.8 \times 10^{-3}$ | 0.06 µg/l (0.17 nM) | $3 \times 10^{-9}$ | (A) Gaido et al., 1997<br>(B) Anderson et al., 1998 |
| 4-Nonyl-phenol | $1 \times 10^{-6}$ | < 1ng/l (4.5 pM) | $1.5 \times 10^{-6}$ | (A) Gaido et al., 1997<br>(B) Müller, 1997 |
| Bisphenol A | $3.4 \times 10^{-6}$ | < 1ng/l (4.4 pM) | $4 \times 10^{-7}$ | (A) Gaido et al., 1997<br>(B) FDA, 1995 |

A) Effective Dose: the lowest reported concentration inducing 50% of the maximum oestrogenic activity in vitro
B) Relative Potency: ratio of concentration in human serum to effective dose, normalized to that of oestradiol

## 3. EDCs and wildlife health

The present literature overview assesses the currently available evidence on adverse health effects on wildlife populations by exposure to environmental contaminants that manifest effects that may be modulated through the endocrine system. Reports of field and semi-field studies dealing with (suspected) endocrine-mediated abnormalities and ecological well-being in both terrestrial and aquatic (fish eating) populations of mammalian, avian, reptilian, fish and invertebrate species are tabulated and discussed. A distinction is made between reproductive and non-reproductive disturbances and/or effects. Particular emphasis in the overview is placed on recent reports from European countries. As a starting point for this review available reviews were used prepared by the Weybridge Workshop of the EC, EEA and WHO (1996) the USA-EPA (1997), the Swedish EPA (1998).

### 3.1 Mammals

#### 3.1.1 Reproductive effects

Effects on reproduction in mammals, in particular reported for the aquatic environment, are summarized in Table 4a.

**Table 4a: Reproductive effects in terrestrial and aquatic (fish-eating) mammals**

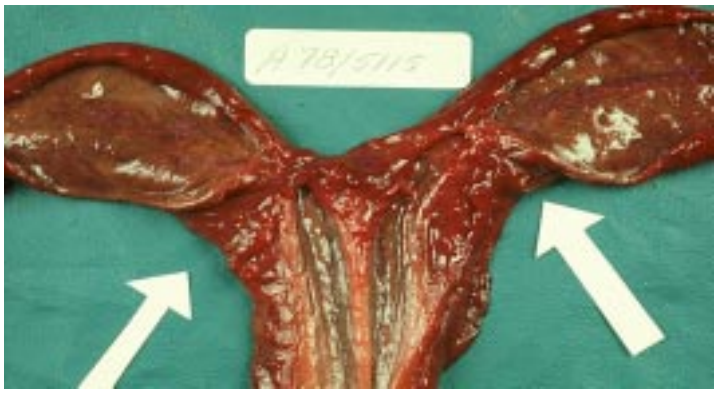
| effect/disorder | location | species | associated contaminants | reference |
|---|---|---|---|---|
| cryptorchidism | Florida | panther (*Felis concolor coryl*) | mercury, *p,p'*-DDE and PCBs | Facemire et al., 1995 |
| masculinisation | Alberta (Canada) | black and brown bear | unknown | Cattet,1988 |
| masculinisation | Spitsbergen | polar bear | PCBs | Wiig et al., 1998 |
| reproductive impairment | Nordic countries | European otter (*Lutra lutra*) | PCBs | Leonards, 1997 |
| reproductive effects | Great Lakes, USA | mink (*Mustrela vison*) otter (*Lutra. canadensis)* | TCDD and related compounds | Gilbertson et al., 1989 |
| premature pupping | California, USA | sea lions (*Zalophus californias)* | DDT-like compounds | DeLong et al., 1973 |
| decreased fecundity, implantation failure | Wadden Sea, NL | harbour seals | PCBs and metabolites | Reijnders, 1986 |
| sterility | Baltic Sea | ringed seals (*Phoca hispida*) | PCBs | Helle, 1980 |
| decreased population size | Lake Samimaa, Finland | Saimaa ringed seal (*Phoca hispida saimensis*) | mercury | Hyvarinen et al., 1998 |
| decreased fecundity, hermaphroditism | St Law. Bay, Canada | Beluga whales (*Delphinapterus leucas)* | PCBs, dieldrin, 2,3,7,8,-TCDD | Martineau et al., 1988; De Guise et al., 1994a |
| reduced testosterone levels | NW Pacific Ocean | Dall's porpoises (*Phocoenoides dalli*) | PCBs and DDE | Subramanian et al., 1987 |

Perhaps the best evidence for cause-effect relationships between organochlorine compounds and reproductive toxicity in a marine mammal comes from the field studies on Baltic grey and ringed seals (see insert 1), and from the semi-field studies on harbour seals, where both reproduction (and immune function; see 3.1.2) have been impaired by PCBs in the food chain (see insert 2). In the semi-field reproduction study two groups, each consisting of 12 female seals, were fed for two years with either a diet of flatfish derived from the Wadden Sea or a diet of mackerel from the open Atlantic. In this prospective study, female harbour seals fed fish from the polluted Wadden Sea

displayed a lower reproductive success than seals fed less contaminated fish from the Atlantic Ocean (Reijnders, 1986). Lower concentrations of 17ß-oestradiol were found in the group with the highest PCB-uptake (Reijnders, 1986, 1996). The reproductive effects observed in the Baltic seals and the harbour seals exposed to Wadden sea flatfish for two years were fairly different. Particularly striking were the reduced fertility, uterine occlusions resulting from abortions, uterine smooth muscle tumours, and a suite of pathological changes including adrenocortical hyperplasia and osteoporosis that characterized the disease syndrome in the Baltic seal populations. In the two different semi-field studies performed with harbour seals decreased fertility, reduced levels of 17ß-oestradiol, retinol and thyroid hormones were found, and various immune function parameters were suppressed that were associated with an increased sensitivity to virus infection. The differences observed between the field study in the Baltic and the Dutch semi-field studies may be explained by the different species (grey, ringed and harbour seals), the different exposure scenarios, as well as the duration of exposure (life-time in the Baltic, two years in the dutch studies). Also the study design and the different biological endpoints examined may explain the different effects observed.

**INSERT 1**

**Baltic seal disease syndrome**



**Uterus from a sterile grey seal showing lesions resulting from a disrupted pregnancy. Arrows depict sites of strictures and occlusions in the uterine horns, originating from aborted early embryos (photo: M Olsson)**

A great deal of attention has been given to a disease syndrome in Baltic grey and ringed seals that is firmly linked to high body-burdens of PCBs, DDT and their persistent methyl sulphone metabolites (Bergman and Olsson, 1985**;** Bergman, 1998; Jensen and Jansson, 1976; Haraguchi et al., 1992). An investigation, initiated because of a serious reduction of the grey and ringed seal populations, revealed that uterine stenosis and occlusions had occurred in 30 % of the adult grey seals and in 70 % of the ringed seals subjected to autopsy (Bergman and Olsson, 1985; Helle et al., 1976a,b, Helle, 1980). In addition, uterine smooth muscle tumours (leiomyomas) were common findings in older grey seals but not in ringed seals (Bergman and Olsson, 1985). Along with decreasing body burdens of organochlorines, the gynaecological health of Baltic seals has gradually improved during the last two decades (Bergman, 1998).

In addition to reproductive disorders and sterility, a suite of pathological lesions have been reported, including severe adrenocortical hyperplasia, osteoporosis, intestinal ulcers and a decreased epidermal thickness in both sexes (Bergman and Olsson, 1985; Bergman et al., 1992; Bergman, 1998, Mortensen et al., 1992; Olsson et al., 1994). Hyperadrenocorticism

(Cushing's disease) has been proposed as one major cause of the disease syndrome in the Baltic seal populations (Bergman and Olsson, 1985). The prevalence of some of these lesions has decreased during the last two decades, especially lesions related to gynaecological health. Notably, however, the incidence of intestinal ulcers seems to be increasing (Bergman, 1998).

Despite a strong correlation to persistent organochlorine exposure, the underlying mode of action and the individual PCB and DDT compounds responsible for these toxic effects in Baltic seals are still incompletely understood. Given the diverse pattern of pathological lesions observed and the large number of organochlorines and metabolites identified in the seals, it is conceivable that the disease syndrome is caused by several classes of persistent organohalogens and several independent mechanisms of action. Since there is often a covariation in the exposure to the different organochlorines, the eco-epidemiological study approach will generally not allow conclusions about the aetiological role of individual chemicals but at most about groups of chemicals. Eco-epidemiology may, however, direct attention to groups of chemicals. Recent studies have thus concluded that high concentrations of DDT compounds were present in reproducing females in the past, whereas females with high PCB concentrations failed to reproduce (Roos et al., 1998). These results support an aetiological role of PCB for parts of the disease syndrome, particularly for lesions connected to the reproductive failure among the seals.

**Transsected adrenal glands from a Baltic grey seal (left) and a grey seal from Svalbard (right) showing massive hyperplasia of the adrenal cortex in the Baltic seal (photo: Swedish Veterinary Institute)**

Several of the observed pathological lesions are compatible with an increased glucocorticoid production in the seals. Osteoporosis, decreased epidermal thickness and immunosupression could result from an increased hormone synthesis in the hyperplastic adrenal cortex. Notably, however, no information on the functional activity of the adrenal cortex in affected animals has been provided. In conflict with the concept of hyperadrenocorticism, recent experimental data rather suggest that several pollutants in the affected seals are capable of disrupting both glucocorticosteroid hormone synthesis and receptor-mediated action. Several distinct mechanisms of action have been described, which could result in a compensatory hyperplasia of the adrenal cortex. Along with a number of methylsulphonyl-PCBs ($MeSO_2$-PCBs), the persistent DDT metabolite $MeSO_2$-DDE was originally identified in blubber of Baltic grey seal (Jensen and Jansson, 1976). Studying the biological properties of this novel class of metabolites, it was found that $MeSO_2$-DDE is a highly potent adrenal toxicant following a site-specific cytochrome P450-catalysed irreversible binding in the glucocorticoid-producing *zona fasciculata* in mice. This observation suggested a role for $MeSO_2$-DDE in the aetiology of adrenocortical hyperplasia in Baltic seals (Lund et al., 1988; Jönsson et al., 1991; 1992; Brandt et al., 1992). A comparative *in vitro* study with adrenal tissue of grey seal, mink and otter showed significant cytochrome P450-catalysed irreversible binding of $MeSO_2$-DDE in

seal adrenal tissue, while no binding of this compound occurred in mink and otter adrenal tissue (Jönsson et al., 1993; Lund, 1994). Another DDT metabolite, the classical adrenocorticolytic agent DDD, showed a marked P450-catalysed binding in the adrenal cortex of seal, mink and otter. Consequently, also DDD could contribute to adrenal dysfunction, resulting in compensatory hyperplasia of the adrenal cortex (Jönsson et al., 1994; Lund, 1994). In addition, $MeSO_2$-DDE and certain $MeSO_2$-PCBs are potent inhibitors of CYP11B1 and corticosterone synthesis in mouse adrenocortical Y1 cells (Johansson et al., 1998a). Finally, as recently reported by the same group, certain $MeS0_2$-PCBs may also act as glucocorticoid receptor antagonists in CHO cells expressing a human glucocorticoid receptor responsive element (Johansson et al., 1998b). This novel finding supports a complex aetiology of disturbed glococorticoid hormone homeostasis in Baltic seals, which could also involve a component of physiological stress.

Cryptorchidism (90% of the male population), high prevalence of sperm abnormalities, and occasional cases of sterility have been reported in the endangered Florida panther (*Felis concolor coryl*) (Facemire et al. 1995). The involvement of endocrine disrupting chemicals in the early developmental stages as a contributing cause for reproductive impairment in this population is suspected, but further studies are required to elucidate the observed reproductive failure and disorders in this population of animals (see EPA, 1997).

Masculinisation (pseudo-hermaphroditism) is reported from local populations of black and brown bears in Alberta, Canada (Cattet, 1988). Recently, a parallel case is reported in polar bears in Europe (Spitsbergen): two female yearlings showed a normal vaginal opening and a 20-mm penis containing a baculum. Since neither of the yearlings showed Y chromosomes, they were considered as female pseudohermaphrodites (Wiig et al. 1998). Polar bears at Svalbard are highly polluted with organochlorines, particularly PCBs (Bernhoft et al. 1997). The authors emphasise that the mechanisms involved are unclear but suggest that the pseudohermaphrodites may be due to endocrine disruption from environmental pollutants.

In top predators such as the mustelids, the European otter (*Lutra lutra*) and mink *(Mustela vision)* high concentrations of PCBs (ppm range) have been reported (Keymer et al. 1988; Giesy et al. 1994a; Leonards, 1997; Wren, 1991). Non-ortho substituted PCBs are generally held responsible for adverse reproductive health effects observed in these species (Aulerlich et al., 1985; Leonards, 1997). In Scandinavia, as well as in many other European countries, the otter populations were found to decline after the 1950s. PCB was early proposed to be responsible for this population decline (Sandegren et al., 1980). The markedly decreased environmental concentrations of PCB recorded in Sweden in the last two decades has consequently been followed by a recovery of the otter populations all over the country. This improvment has been most pronounced in the remote, less contaminated parts of Sweden (Roos et al., submitted).

Today thriving otter populations are found only at the boundaries of Europe where lowest concentrations of PCBs are present (Leonards, 1997). There is however one exception, the Shetland islands, where relatively high concentrations of PCBs are found in a thriving otter population (Kruuk and Conroy, 1991). A literature review of the presence of PCBs in otters (Smit et al. 1994) concluded that it was neither possible to dismiss the role of PCBs in the decline of otter populations nor to assume the role as completely proven. This was mainly due to the fact that reported concentrations of PCBs in otters are difficult to compare because of differences in analytical methods.

Several experimental studies have revealed a high reproductive toxicity of PCB (but not of DDT) in mink (Aulerich and Ringer, 1985; Jensen et al., 1976; Kihlström et al., 1992; Brunström et al.,

1998). Mink exposed to a technical PCB preparation (Clophen A50) for 18 months showed a dose-dependent decrease in the number of whelps born with virtually no reproductive capacity remaining in females exposed to 0.3 mg PCB/day (Brunström et al., 1994). Based on a sensitive EROD bioassay, the highest amounts of 'dioxin-like' compounds (Bio-TEQs, 2,3,7,8-TCDD toxic equivalents) found in Swedish otters were higher than those in the Clophen A50-exposed mink (Brunström et al., 1998). Assuming a similar reproductive toxicity of PCB in otter as in mink, these results clearly support the conclusion that PCB exposure may be involved in the decline of European otter populations.

Toxicity data and levels of PCBs observed in wildlife populations of mink (*Mustrela vison*) and otters (*L. canadensis*) in the Great Lakes Basin (USA) were reviewed by Wren (1991). He concluded that there was little doubt that contaminants had effects on mink and otters from the Great Lakes Basin, but that available data were insufficient to provide final proof of cause-effect linkages between chemical compounds and population status. Data on contaminants in the diet of mink and otter were not available.

A large number of xenobiotics with potential endocrine disrupting effects have been reported in tissues of many marine mammals (Colborn and Smolen, 1996; Reijnders, 1996, Wagemann and Muir, 1984). In general these species are related to coastal zones. However, more recently it has been shown that tissue of more oceanic species such as sperm whales (*Physeter macrocephalus)* and minke whale *(Balaena acutorostrata)*, show considerable concentrations of these compounds including toxaphene and polybrominated diphenylethers (PBDE) (de Boer et al., 1998). Reproductive and developmental disorders in these mammals have been associated with certain organochlorine contaminants (mainly DDT metabolites and PCBs, and their metabolites) in Californian sea lions (*Zalophus californias)* (DeLong et al. 1973, Addison, 1989), ringed seals (*Phoca hispida*) and grey seals (*Halichoerus grypus)* in the Baltic Sea (Helle, 1980; Bergman and Olsson, 1985), harbour seals (*Phoca vitulina*) in the Wadden Sea (Reijnders, 1986), and beluga whales (*Delphinapterus leucas)* from the polluted Gulf of St Lawrence (Beland et al. 1987; Martineau et al. 1988) (See Table 3a). In most of the above cases, however, it was not possible to confirm a cause and effect relationship between a specific chemical and effects found (Reijnders and Brasseur, 1992; Colborn and Smolen, 1996; O'Shea and Brownell, 1998). In all of the above cases, the etiology of the observed effects remains therefore unresolved (Reijnders and Dijkman, 1993).

Decreased testosterone levels in the blood have been associated with high concentrations of PCBs and DDE in the blubber of Dall's porpoises (*Phocoenoides dalli*) from the Northwest Pacific, but the mechanism for this decrease remains unknown (Subramanian et al., 1987). It can be speculated that PCB-related enzyme induction, as proposed for seals in the Wadden Sea, may have played a role.

High levels of organochlorines found in beluga whales from the polluted Gulf of St Lawrence in Canada have been associated with the low reproductive rate of the population (Martineau et al., 1988). In addition, a single unique case of true hermaphroditism (presence of ovaries and testes) was described in one out of nine investigated beluga whales from this area (De Guise et al. 1994a). However, based on the available literature and most recent findings, the evidence for a primary effect of contaminants leading to the observed effects in St Lawrence belugas has been considered largely circumstantial, and should be treated with caution (ICES, 1998).

In a very recent report of the Saimaa ringed seal (*Phoca hispida saimensis*) in Lake Saimaa, Finland it has been suggested that increased mercury levels in the seal pups, possibly due to selenium

shortage, may have contributed to the decrease in population density of this population in the 1960s and 1970s (Hyvarinen et al., 1998).

**INSERT 2**

**PCBs, PCDFs and PCDDs as cause of reduced reproduction and suppressed immunity in harbour seals**

**Reproduction study**

The best evidence in marine mammals for a causal link between endocrine disruption and exposure to organochlorines comes from the semi-field studies on Wadden Sea harbour or common seals *(Phoca vitulina)*, where both reproduction and immune functions have been impaired. Reproduction effects resulted in population declines, whereas suppression of immune function have likely contributed to the mass mortalities due to morbillivirus infections.

In the reproduction study, two groups, each consisting of 12 female seals, were fed for two years with either a diet of flatfish derived from the Wadden Sea or a diet of mackerel from the open Atlantic. In this prospective study, female harbour seals fed fish from the polluted Wadden Sea displayed a lower reproductive success than seals fed less contaminated fish from the Atlantic Ocean (Reijnders, 1986). In the same study, the Wadden Sea group seals also had lower concentrations of 17β-oestradiol (Reijnders, 1986). Two possible explanations for the low reproductive success are given by Reijnders (1996). Firstly, an increased breakdown of steroid hormones and consequently lower plasma concentrations as a consequence of cytochrome P450 (CYP1A) enzyme induction by PCBs/PCB-metabolites. This process has been shown in various other studies (Tanabe et al., 1988; Boon et al., 1992). However, because Froixi and Mason found a negative correlation between PCB concentrations and hormone metabolism, this mechanism seems to be a less likely explanation (Reijnders, in press 1998). Secondly, interference by PCB or DDE and their metabolites with receptors in target tissues could be involved. PCB-methylsulphones bind with high affinity to a uteroglobin-like protein in the murine and human airways (Brandt et al. 1995; Lund et al. 1995). Uteroglobin is a steroid-binding protein that was originally identified in secretions from the uterus and uterine tubes and probably plays a regulatory role in the reproductive tract. Considering the above reports on uterine strictures and occlusions in Baltic seals, it was of particular interest that phenolic and methylsulphone-containing PCB metabolites are selectively enriched in the uterine luminal fluid of pregnant mice (Brandt et al. 1982).

Seals from the group with the highest PCB-uptake had reduced levels of retinol (vitamin A) and thyroid hormones (Brouwer et al., 1998), both biomarkers generally associated with exposure to PCBs and related compounds (Peakall, 1993). Competition between thyroid hormones and retinol and certain PCB congeners (such as PCB-77) for binding to a transport protein was presumably responsible for the reduced plasma levels (Brouwer et al., 1989). It is known that reduced levels of retinol and thyroid hormones may be of biological significance. Thyroid hormones play an important role in the development of the brain during the early developmental period, and an indirect role in the maturation of the testicular Sertoli and Leydig cells, which are important in spermatogenesis. The latter is known to be important in cell differentiation, while vitamin A deficiency may lead to delayed growth and reduced reproductive success. Based on the presence of hydroxylated PCB metabolites in seal blood (Bergman et al., 1994) and the strong accumulation of such metabolites in the feto-placental unit in pregnant mice (Brandt et al. 1982, Darnerud et al. 1986), it has been postulated that

foetal accumulation of PCB metabolites occurs also in pregnant seals (Murk et al., 1994; Brouwer et al., 1998).

**Harbour and grey seals at Blakeney Point, Norfolk, United Kingdom (photo: JG Vos)**

**Immune function study**

The mass mortalitiy of harbour seals in northwestern Europe in 1988 resulting from infection with a previously unidentified morbillivirus (Osterhaus and Vedder, 1988), being subsequently characterized and named phocid distemper virus 1 (PDV-1), led to speculation about the possible contributing role of environmental comtaminant-induced immunosuppression (Dietz et al., 1989). A similar epizootic among Baikal seals *(Phoca sibirica)* in Siberia in 1987 was later attributed to infection with canine distemper virus (Grachev et al., 1989; Osterhaus et al., 1989). In order to investigate the role of immunotoxic environmental pollutants, including the PCBs, PCDFs and PCDDs (Vos and Luster, 1989; Vos et al., 1991), a semi-field study was carried in which two groups of 11 harbour seals were fed herring (*Clupea harengus*) from either the highly contaminated Baltic Sea or the relatively unpolluted Atlantic Ocean. A variety of parameters related to immune function were monitored and compared in the two groups over a period of 2 year. Natural killer-cell activity and T-cell mitogen (but not B-cell)-induced proliferative responses in peripheral blood were found to be significantly lower in the seals fed contaminated fish (De Swart et al.,1994). While the results of these non-specific *in vitro* tests of immune function indicated that contaminants in the Baltic herring were immunotoxic, further evidence was provided when impaired mixed lymphocyte reactions and antigen-specific lymphocyte proliferative responses were observed in the Baltic group (De Swart et al., 1995). In addition, *in vivo* antibody responses and delayed-type hypersensitivity skin reactions were significantly depressed following immunisation with ovalbumin, both being T-cell dependent immune responses (Ross et al., 1995, 1996a). The observed immunsuppression clearly indicates that in harbour seals the resistance to a virus infection is impaired as NK-cells and T cells play a major role in defence against viral infections. The impaired immune responses in seals fed the Baltic Sea herring are consistent with the effects observed in studies of laboratory animals exposed to Ah-receptor binding PCBs, PCDFs and PCDDs (Vos and Luster, 1989; Vos et al., 1991; Vos et al., 1997/98). An additional indication that PCB-like compounds led to a toxic effect in the seals of the Baltic group was the observed decline in vitamin A levels (De Swart et al., 1994). The estimated daily intakes of 2,3,7,8-TCDD toxic equivalents (TEQs) by the Baltic group of seals were 10 times higher than those of the Atlantic group of seals (De Swart et al., 1984), and led to a blubber concentration of 286±17 ng TEQ/kg lipid (mainly derived from mono-ortho PCB) in the Baltic seals compared to 90±6 ng TEQ/kg lipid in the Atlantic seals (Ross et al., 1995). From the comparison of these results and literature data it appears that many

free-ranging harbour seal populations inhabiting polluted areas of Europe and North America have ΣPCBs or TEQ blubber concentrations being at or above those observed in the Baltic group of seals of the semi-field study (Ross et al., 1996b). Chemical-induced immunosuppression may therefore, besides its contribution to the severity and extent of the 1988 morbillivirus outbreak, affect the immunocompetence of free-ranging populations in many areas of the industrialized world. This is the more true as free-ranging seals are likely to be more vulnerable than the seals of the semi-field study as perinatal TCDD exposure produces more profound immunosuppression, at least in laboratory rodents (Vos and Luster, 1989), and the lifespan of seals allows for long-lasting accumulation (Ross et al., 1996b). Finally, it is likely that hormone disruption is involved in the observed immunosuppression, as TCDD causes thymic atrophy that is linked to an adverse effect on the epithelial cells of the thymus, which cells support thymocyte proliferation and maturation by cell-cell contact and secretion of thymic hormones (De Waal et al., 1997).

### 3.1.2 Non-reproductive effects

Besides poor reproductive performance, also mass mortalities by infectious diseases, immunosuppression, thyroid abnormalities and other non-reproductive disorders have been associated with the presence of organochlorine compounds and other potential endocrine disrupting chemicals in body fat of mammals (Table 4b).

In addition to reproductive disorders and sterility in Baltic and North Sea seals described in 3.1.1**,** a suite of other pathological disorders have been reported, including severe adrenocortical hyperplasia and osteoporosis; and MeSO2-DDE was causally linked to the adrenocortical hyperplasia in Baltic seals (see insert 1). The role of chemical-induced immunosuppression in the mass mortality in 1988 in harbour seals due to morbillivirus infections has been investigated in a second semi-field study by Dutch research groups. In this long-term feeding study it is shown that ambient levels of environmental contaminants, notably PCBs, are immunotoxic to harbour seals. It is concluded that the morbillivirus infection in harbour seals was exacerbated by these contaminants (see insert 2).

The health status of Danish otters (*Lutra lutra*) was investigated in relation to the concentration of PCBs on a TEQ basis (Leonards et al., 1998). The concentration range showed an association with increased rates of various skin and internal lesions including infections and correlated well with decreased levels of hepatic retinol or retinylpalmitate found in the otter. These results indicate that the concentrations of PCBs found in European otters could be associated with adverse health effects in the individual animal. Any consequences at the population level are yet unknown.

Two experimental studies have provided evidence for immune modulation from dietary exposure to bleached-kraft pulp mill effluent (BKME) in mink. Mink exposed for 26 weeks to dietary BKME responded with a significantly greater antibody reponse following vaccination with mycobacterium than did control mink (Smits et al., 1996). By contrast, their cell-mediated immune response was suppressed (Smits et al., 1996).

**Table 4b: Non-reproductive effects in terrestrial and aquatic (fish-eating) mammals**

| effect/disorder | location | species | associated contaminants | reference |
|---|---|---|---|---|
| skull lesions | Baltic Sea;<br>German, Wadden Sea | harbour seals<br>(*Phoca vitulina*) | PCBs<br>DDT/DDE | Mortensen et al., 1992;<br>Stede and Stede, 1990 |
| uterine stenosis, occlusions | Baltic Sea | ringed seals<br>(*Phoca hispida*) | DDE-/PCB-methylsulfones | Helle et al., 1976b<br>Olsson et al., 1994 |

| | | | | |
|---|---|---|---|---|
| | | | | Bergman, 1999 |
| increased rates of lesions and infections lowered levels of hepatic retinol or retinylpalmitate | Denmark | European otter (*Lutra lutra*) | PCBs | Leonards et al., 1998 |
| lowered levels of vitamin A and thyroid hormones | Netherlands, Wadden Sea, | harbour seals (*Phoca vitulina*) | PCBs | Brouwer et al., 1989 |
| skull-bone lesions (osteoporosis) | Baltic Sea | grey seals (*Halichoerus grypus* | PCBs, DDT/DDE | Bergman et al., 1992 |
| adrenocortical hyperplasia | Baltic Sea | ringed seals (*Phoca hispida*) grey seals (*Hali-choerus grypus)* | PCBs | Bergman and Olsson, 1985; Olsson et al., 1994 |
| lowered immuno-competence | Netherlands, Wadden Sea | harbour seals (*Phoca vitulina*) | TCDD-like PHAHs | Ross et al., 1995; Swart et al., 1994 |
| mass mortalities | USA, Atlantic Ocean, Mediterranean Sea | various dolphin species | *o,p'*-DDE, *p,p'*-DDE *o,p'*-DDT, PCBs | Lahvis et al., 1995 Geraci, 1987 Aguilar, 1994 |
| mass mortality | Mediterranean Sea | striped dolphins *(Stenella coeruleoalba)* | TBT? | Scott et al., 1988 |
| pathological disorders | Canada, Gulf of St Lawrence | Beluga whales (*Delphinapterus leucas)* | organochlorines | De Guise et al., 1994b |

High levels of organochlorines have been noted in dolphins which died in several mass mortality incidents. Aguilar (1994) compared PCB levels in striped dolphins *(Stenella coeruleoalba)* which died during the Mediterranean morbillivirus epizootic in 1990 with biopsies taken from live dolphins in 1987-89. These authors suggested that PCBs left the blubber during mobilisation of fat reserves and large quantities reached the liver shortly before the epizootic. This may have increased the dolphins' susceptibility to the morbillivirus.

Kuiken et al. (1993) compared PCB levels in by-caught porpoises and those that died from disease, and found no difference in levels. They, highly disputably, inferred from this finding that PCBs were not suppressing the immune system in these animals from British waters.

Mass mortality of bottlenose dolphins (*Tursiops truncatus*) along the east coast of America in 1987-88 have been associated with consumption of fish poisoned with brevetoxin - a naturally occurring algal toxin (Geraci, 1987). However, various other authors have disputed this conclusion and suggested that the high concentration of organochlorines found in the carcasses were a major factor in the mortality (Lahvis et al., 1995). The dolphins also showed a number of symptoms indicative of immune system dysfunction (skin and organ lesions, most of them believed to be caused by opportunistic infections by bacteria, viruses and fungi) which were positively correlated with various organochlorine compounds including DDT and PCBs (Lahvis et al., 1995).

High concentrations of butyltin residues have found in harbour porpoise from the Baltic Sea (Poland) and from bottlenose dolphins from the Mediterranean (Kannan and Falandysz, 1997). Organotin compounds (bis(tri-*n*-butyltin)oxide) have been shown to exhibit immunosuppressive and endocrine modulating activities in rodents (Vos et al., 1984; Krajnc et al., 1984). In particular long-term exposure reduced the resistance to infectious diseases (Vos et al., 1990)

Beluga whales from the Gulf of St Lawrence in Canada show a high prevalence of lesions of endocrine glands (hyperplastic and degenerative changes of adrenals) (Lair et al. 1994, 1997) and thyroids (in ICES, 1998). Similar changes were not observed in belugas from less contaminated areas (Lair et al. 1997, St. Aubin and Geraci, 1989) and in other species of marine mammals (Cowan 1967; Kuiken et al. 1993). In addition to the above mentioned pathologies, frequent infection by mildly pathogenic bacteria and more importantly a high prevalence of various types of tumours have been observed in these stranded animals (Martineau et al., 1994, De Guise et al., 1994b). The prevalence of malignant tumours in belugas from the St. Lawrence estuary is high and affecting mainly older animals (17 years and older).

## 3.2 Birds

### 3.2.1 Reproductive effects

Reproductive effects have been reported in many species, especially aquatic and terrestrial birds of prey (Table 5).

PCBs were originally identified as environmental pollutants in a white-tailed sea eagle *(Haliaeetus albicilla )* found dead in the Stockholm archipelago (Jensen 1966, 1972). The reproductive success of the Baltic sea eagle was subsequently shown to be negatively correlated to the levels of persistent organochlorines in their eggs (Helander et al., 1982; Falandysz et al., 1994).

Bird eggs are important matrices for monitoring the levels of persistent pollutants in aquatic and terrestrial ecosystems, and many lipophilic chemicals such as PCBs, PCDFs, PCDDs, chlorinated pesticides are readily taken up and excreted in the egg yolk in laying birds. This efficient route of elimination from the laying female may result in a pronounced exposure of the avian embryo to toxic chemicals from its earliest stages of development. Certain avian top predators may therefore be sensitive targets for these persistent lipophilic pollutants.

Several examples of pollutant-induced toxicity in wild bird populations and reproductive or developmental toxicity following exposure to chlorinated pesticides, PCBs, PCDFs and PCDDs have been reported (Gilbertson et al., 1991; Fry, 1995, and references therein). In birds as in mammals, it is generally difficult to determine the mechanism of action in the field, where toxic effects may result from co-exposure to complex mixtures of persistent organic pollutants and metals. Based on recent experimental data, those chemicals characterised to date may affect the endocrine system by a number of mechanisms including oestrogen-, androgen- thyroxin- glucocorticoid- and retinoid-regulated pathways. In addition, Ah-receptor agonists such as PCDDs/PCDFs, coplanar PCBs and polycyclic aromatic hydrocarbons (PAHs) represent an important group of highly toxic compounds with an array of toxic effects including reproductive toxicity and teratogenicity (Bosveld and Van den Berg, 1994). Some endocrine disrupters may work by different mechanisms of action in different tissues and species, as demonstrated by the classical DDT-molecule and its persistent metabolites DDE, DDD and methylsulphonyl-DDE (Brandt et al., 1997).

**Table 5: Reproductive effects in birds**

| effect/disorder | location | species | associated contaminants | reference |
|---|---|---|---|---|
| reproductive failure | Sweden, Baltic Sea | white-tailed sea eagle | PCBs, DDT | Jensen, 1972; Helander et al., 1982 |
| eggshell thining | Europe, North America | numerous species, e.g. perigrins, osprey, bald eagle, sparrow hawk, guillemot | DDE | Ratcliffe, 1967 Koeman et al., 1972 Peakall, 1973 Bignert et al., 1995 Odsjo and Sondell, 1982 |
| reproductive failure | Sweden, Netherlands | pelegrim, sparrow hawk?? | pesticides, methylmercury | Borg, 19xx, Koeman et al., 1972 |
| behavioural effects | Sweden | robin | Clophen | Ulfstrand et al., 1971 |
| feminization, ovotestis, persistent Mullerian ducts, female- female pairing | USA, California, Great Lakes | Western gull | DDT compounds, methoxychlor, OC | Fry, 1995; Fry and Toone, 1981 |
| reproductive failure, deformities | USA, Great Lakes | colonial fish eating birds | PCDD/PCDF | Gilbertson et al., 1991 Giesy et al, 1994b |
| embryotoxicity, impaired adrenal and ovarian function | oil spill contaminated seas | sea birds | PAH fractions | Hoffman, 1990 Rattner et al., 1984 |
| decreased hatching success | USA, Lake Michigan | Forster's tern *(Stema forsteri)* | TCDD, PCDD, PCB's, DDT, DDE | Kubiak et al. 1989 |
| reproductive impairment | USA, Europe | numerous species | PCDDs, PCDFs, PCBs | Hoffman et al., 1996 Hart et al., 1991, |
| female-female pairing eggshell thinning | USA, California, Great Lakes, Puget Sound, | Western gull | DDT compounds | Fry et al ., 1987 |
| goiter and thyroid abnormalities | USA, Great Lakes | herring gull *(Larus argentatus)* | PHAHs? | Moccia et al. 1986 |
| developmental impairment | The Netherlands, river Rhine and its tributaries | cormorant (*Phalacrocorax carbo*) | dioxins and related compounds, PCBs, DDE | Boudewijn et al. 1988, Dirksen et al., 1995; Van den Berg et al. 1994 |
| lower breeding success poor survival of young | The Netherlands, Haringvliet, semi-field study | tufted duck (*Aythya fuligula*) | heavy metals and PCBs | Marquenie et al. 1986 |
| eggshell thining breeding failure population declines | USA, Great Lakes | seabirds | DDT/DDE | Cooke, 1973 |
| impaired reproduction | San Fransico Bay, Great lakes | crowned night heron *(Nycticorax nycticorax)* | PCBs | Hoffman et al. 1996 |
| population decline reduced growth and behavioural activity | semi-field conditions, Wadden Sea, NL | eider duck (*Somateria mollissima*) | Ah-receptor-related compounds, PCB-77, Clophen A50 | Murk et al. 1994a Swennen, 1991 |
| reduced hatchability, dead chicks, morphological abnormalities | Dutch and Belgian estuaries, Western Scheldt | common tern *(Stema hirundo)* | PHAHs? | Murk et al. 1994b, 1996 Bosveld et al. 1995 |

DDE-induced reproductive failure due to eggshell thinning is one of the most serious and well-documented effects of persistent organic pollutants in avian wildlife (see insert 3).

**INSERT 3**

**DDE-induced eggshell thinning**

Ratcliffe (1967) drew attention to the thinning of the eggshells combined with the occurrence of broken eggs that had been observed in nests of the peregrine falcon (*Falco peregrinus* ), sparrowhawk (*Accipiter nisus*), and golden eagle (*Aguila chrysaetos*) since about 1946 in Great Britain. He noted that that at the same time DDT had come into general use. As shown by numerous subsequent experimental and field studies, eggshell thinning may be produced by several environmental pollutants including methyl mercury and organochlorines such as the persistent DDT metabolite DDE. Notably, *p,p'*-DDE is the dominating DDT-residue in most bird species. DDE-induced reproductive failure due to eggshell thinning and broken eggs is one of the most serious and well-documented effects of persistent organic pollutants in avian wildlife (Ratcliffe, 1970; Peakall et al., 1973; Lundholm, 1997). It is probably also the first example of endocrine disruption in wild populations, even though the mechanism of action has not been understood until recently.

Originally reported in peregrins, eggshell thinning has been described in a large number of species including raptors such as the osprey *(Pandion haliaetus)* and the bald eagle *(Haliaetus leucocephalus)* (Koeman et al., 1972; Lincer, 1975; Odsjö and Sondell, 1982; Peakall et al., 1973; Ratcliffe 1970). Bignert et al. (1995) examined eggshell thickness in Baltic guillemot *(Uria aalge)* eggs collected 1861-1989. Along with decreasing DDT and PCB levels in the Baltic fauna since the 1970s, the eggshell thickness in Baltic guillemot has gradually improved. Notably, however, guillemot eggshells were still thinner in their recent material than in historical material collected before 1946.

**Measuring the shell thickness of osprey egg**
**(photo: Swedish Natural Museum)**

Several hypotheses have been forwarded to explain the mechanism of eggshell thinning. Studying the pigeon (*Columba livia*), Peakall (1970) reported that *p,p'*-DDT decreases both oestradiol blood levels and deposition of medullary bone, which is an important source of

calcium for the formation of the eggshell. Another proposed mechanism involves inhibition of carbanhydrase in the eggshell gland following exposure of Japanese quail (*Coturnix japonica*) to *p,p'*-DDT and *p,p'*-DDE (Bitman et al., 1970). Carbanhydrase supplies carbonate ions required for calcium carbonate deposition in the eggshell, and the carbanhydrase inhibitor acetazolamide produces experimental eggshell thinning in chicken (*Gallus domesticus*). Despite these early proposals, the mechanism of *p,p'*-DDE-induced eggshell thinning has not been clarified until recently. Using the domestic duck as the test organism, Lundholm (1997) showed that *p,p'*-DDE inhibits prostaglandin synthase and reduces prostaglandin E2 in the eggshell gland mucosa, resulting in a reduced calcium transport across the mucosa into the eggshell gland lumen. The structurally related compounds *o,p'*-DDE, *p,p'*-DDT, *o,p'*-DDT, and *p,p'*-DDD did not cause eggshell thinning or inhibit prostaglandin synthesis in the eggshell gland. The finding that eggshell thinning could also be induced by the cyclooxygenase inhibitor indomethacine suggested that inhibition of prostaglandin synthesis was part of the mechanism of action (Lundholm 1997).

Experimental studies have shown that in ovo exposure to xeno-oestrogens such as *o,p'*-DDT, DES and 17α-ethinyloestradiol may alter male sexual behaviour in adult birds (Bryan et al., 1989; Berg et al. 1998). Changed behavioural patterns have been observed also in wild birds, even though the mechanisms are not well understood. Hunt and Hunt (1973, 1977) noted female pairing in gulls in southern California when the sexes of birds producing supernormal clutches were examined. Gulls normally lay 3 eggs whereas numbers of eggs in the supernormal clutches were 4-6. Supernormal clutches and female-female pairing have been noted in breeding populations of four species of gulls in North America. Western gull *(Larus occidentalis)* and herring gull *(Larus argentatus)* colonies with supernormal clutches have been located in areas contaminated with high levels of organochlorines. Fry and Toone (1981) suggested that female-female pairing among Western gulls in southern California was related to high organochlorine levels. Since gulls seem to be rather resistant to eggshell thinning by DDT, levels of DDT causing eggshell thinning in other species such as the brown pelican *(Pelecanus occidentalis)* did not damage shells of gull eggs. Hence, gull chicks hatched from eggs containing high levels of organochlorines.

Experimental injection of the DDT derivatives *o,p'*-DDT (an oestrogen in rats), methoxychlor (an oestrogen following demethylation in rats) and *p,p'*-DDE (an antiandrogen in rats) into embryonated gull eggs resulted in malformed and feminised sex organs in male chicks. Similar effects were observed when oestradiol was injected as a positive control (Fry and Toone, 1981). All substances induced primordial germ cell growth in an increased, ovary-like cortex of the left testicle (ovotestis) and retention of the left or both Müllerian ducts. Notably, these changes were observed at environmentally relevant doses. These authors also speculated that reproductive failures, skewed sex ratios, and female-female pairing in breeding populations of Western gulls on the Great Lakes resulted from pollution with organochlorines. Feminisation of male gull embryos was suggested to affect reproductive behaviour in males leading to reduced migration to the breeding sites. A reason for female-female pairing in a population could be a skewed sex ratio with an increased number of females. An alternative hypothesis to explain the sex ratio skew in contaminated areas is that the low male/female ratio was due to a higher mortality among the males (Conover and Hunt, 1984).

Colonies of glaucous-winged gulls *(Larus glaucescens)* breeding in polluted areas of Puget Sound in 1984 exhibited eggshell thinning and persistent right oviducts in adult females (Fry et al., 1987). Twenty-one of 31 female gulls retained right oviducts of which 13 were longer than 10 mm. Normally, female birds have completely regressed or small (less than 5 mm) right oviducts. The causes of these effects in the glaucous-winged gulls are unknown. The major contaminants in Puget

Sound were heavy metals, PCB and polycyclic aromatic hydrocarbons, whereas DDT concentrations were not particularly high.

Several species of colonial fish-eating birds nesting in the Great Lakes basin have exhibited chronic impairment of reproduction (Gilbertson et al., 1991; Kubiak et al., 1989). High levels of DDT and its metabolites have caused eggshell thinning in many species. In addition, the reproductive impairment is characterised by high embryonic and chick mortality, oedema, growth retardation, and deformities as well as altered incubation behaviour contributing to decreased hatchability. The syndrome of fish-eating birds in the Great Lakes has been designated GLEMEDS (Great Lakes Embryo Mortality, Edema, and Deformities Syndrome) and clearly resembles chick oedema disease, a condition resulting from unintended exposure of millions of broilers to 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin in south-eastern United States in 1957 (Firestone, 1973). Reproductive impairment related to high PCDD/PCDF exposure has been described in a number of species including the double-crested cormorant *(Phalacrocorax auritus)*, black-crowned night heron *(Nycticorax nycticorax)* , herring gull *(Larus argentatus)*, ring-billed gull *(Larus delawarensis)*, common tern *(Stema hirundo)*, Forster's tern *(Stema forsteri)*, and Caspian tern *(Hydroprogne caspia)*. Current concentrations of PCDDs/Fs and PCBs in fish-eating birds in the Great Lakes area are less than those during the 1960s and 1970s and some bird populations such as double-crested cormorants and herring gulls have made dramatic recoveries since that time (Giesy et al., 1994b). Certain populations of common and Forster's tern continue to decline, however, and double-crested cormorants and Caspian terns in Saginaw and Green Bays still display abnormal rates of developmental deformities and embryo lethality (Giesy et al., 1994b).

No thorough investigation of the possible occurrence of GLEMEDS-like symptoms in fish-eating bird populations in Europe seems to be available, even though TCDD-receptor binding PCBs, PCDDs and/or PCDFs have been found to be present in biologically active concentrations in several wild bird populations at the North Sea coast, in the Baltic area or the Norwegian Arctic (Bosveld and Van den Berg, 1994; Bernhoft et al., 1997; Falandysz et al., 1994). Van den Berg et al. (1994) examined correlations between PCB, PCDD and PCDF levels in the yolk sac, and a number of biochemical and morphological changes in newly hatched chicks from two Dutch cormorant (*Phalacrocorax carbo*) colonies. Significant concentration-effect relationships for EROD induction, plasma free thyroxine reduction, yolk sac weight, relative liver weight and head size were observed. The authors conclude that these alterations may play a role in the observed reduced reproductive success of cormorants in the Dordtse Biesbosch area. Artificial incubation of common tern *(Stema hirundo)* eggs collected in the main sedimentation area of the Rhine and Meuse rivers resulted in a four-fold increase in EROD activity in the developing chick liver as compared to chick livers from eggs collected in a reference colony (Bosveld et al., 1995). Residual yolk sacs from the hatchlings contained on an average 16 ng TEQ (TCDD toxic equivalents) per g lipid, a concentration five times higher than that in the reference colony, and the average TEQ concentrations in chicks hatched after 23 days of incubation were twice the concentrations found in chicks that hatched after 21 days of incubation (Bosveld and Van den Berg, 1994). Murk et al. (1996) examined the relation between yolk sac polyhalogenated hydrocarbon levels and reproductive outcome using embryonated eggs collected from eight Belgian or Dutch common tern colonies. The results showed that higher yolk sac polyhalogenated hydrocarbon levels and liver EROD levels correlated with late egg laying, prolonged incubation period, small eggs and chicks. These parameters also correlated with low yolk sac retinoid and plasma thyroid hormone levels in the embryos.

Extractable lipid tissue from glaucous gull *(Larus hyperboreus)* caught in the Norwegian Arctic (Svalbard) has been found to contain PCB in the 10-1000 ppm range (Gabrielsen et al., 1995). Using a sensitive bioassay based on cultivated chicken embryo liver to estimate the amounts of 'dioxin-like' compounds in captured gulls fed on polar cod *(Boreogadus saida)*, Henriksen et al.

(1998) showed that the Bio-TEQs ranged from 5-254 ng/g extracted lipid. Similar levels of TCDD or TCDD-equivalents in eggs have been associated with embryotoxicity in other bird species (Hoffman et al. 1996).

Bird embryos are highly sensitive to petroleum. Small quantities (1-20 u1) of some types of oil on the eggshell are sufficient to cause embryo death, particularly during early life-stages. The PAH fractions of crude and refined oils seem to be responsible for the lethal and sublethal effects on bird embryos caused by eggshell oiling (Hoffman, 1990; Parnell et al. 1984). Crude oil also exerts effects on the adult birds such as ultrastructural changes in the adrenals, diminished adrenal responsiveness to adrenocorticotrophic hormone and reduced corticosterone secretion, suggesting a direct effect of petroleum on the adrenal gland. Suppression of ovarian function and decreased circulating prolactin levels caused by oil exposure have been associated with impaired reproductive function.

### 3.2.2 Non-reproductive effects in birds

Several phenolic environmental pollutants bind with high affinity to the thyroxin transport protein transthyretin in rodents. This competitive binding provides a mechanism for disruption of thyroxin transport to the tissues. Recent studies have shown that phenolic metabolites of persistent halogenated hydrocarbons such as PCBs are present in blood plasma of Laysan and black-footed albatrosses (*Diomedrea immutabilis; Diomedrea nigripes*) collected at the Midway islands (Klasson-Wehler et al., 1998). The impact of such phenolic pollutants on thyroxine homeostasis in birds remains to be established.

The ubiquitous DDT metabolites 3-methylsulphonyl-DDE and DDD are metabolism-activated toxicants in the corticosteroid-producing interrenal cells in chicken embryos (Jönsson et al., 1994). Information on the effects of these compounds on interrenal cell function in wild birds is not available.

## 3.3 Reptiles/amphibians

### 3.3.1 Reproductive and non-reproductive effects

Reproductive and non-reproductive effects have been reported in various species of aquatic reptiles and amphibians (Table 6).

Research has shown that all crocodiles, many turtles and some lizards lack distinct sex chromosomes and that the sex is not organized until well after fertilization, during organogenesis. In these animals gender is determined during organogenesis by incubation temperature - temperature dependent sex determination (TSD) (Crews, 1994; Crain and Guillette, 1998). Experimental evidence show that animals with TSD are sensitive to developmental exposure to exogenous hormones, and temperature-dependent gender can be altered by hormone exposure (Crain and Guillette, 1997). When 17β–oestradiol is administrated to eggs during the temperature sensitive period, phenotypic females are produced at male-producing temperatures in turtles, lizards and alligators (see Crain and Guillette, 1998).

Experimental studies in turtles that exhibit TSD show sex reversal following embryonic exposure to various polychlorinated biphenyls. For instance, Bergeron et al. 1994 found that exposure of red-eared slider turtle (*Trachemys scripta elegans*) eggs to artificial hydroxy-PCBs (2,4,6-trichloro-4'-biphenylol and 2,3,4,5-tetrachloro-4'-biphenylol), incubated at a temperature known to produce 100% males, significantly altered sex ratio, producing more females. The results

were identical for oestradiol-treated eggs. There was no evidence of sex reversal for (combined) nonhydroxylated PCBs. Results of this laboratory study indicate that also hydroxy-PCBs found to be present in the blood of wild mammals, birds and fish (Bergman et al., 1994; Klasson-Wehler et al., 1998; Asplund et al., 1999) may have the capacity to exert oestrogenic effects on the embryonic gonad and thus influence sexual differentiation in wildlife.

**Table 6: Reproductive and non-reproductive effects in reptiles and amphibians**

| effect/disorder | location | species | associated contaminants | reference |
|---|---|---|---|---|
| decreased hatching success, increased developmental abnormalities | Great Lakes | snapping turtles *(Chelydra serpentinia serpentinia)* | organochlorine compound including PCBs, PCDDs, DDE and other pesticides | Bishop et al., 1991 |
| feminization | Canada Lake Ontario | snapping turtles *(Chelydra serpentinia serpentinia)* | organochlorine compounds | De Solla et al., 1998 |
| low hatching rates, various abnormalities in males and female, including abnormal gonadal morphology, altered gonadal steroidogenesis, and abnormal sex steroid concentration | Florida, USA | American alligator *(Alligator mississippiensis)* | p,p- DDE | Guilette et al, 1994, 1996 |
| inhibition of metamorphosis and high prevalences of skin lesions | USA, lagoon | salamander (*Ambystoma tigrinum)* | pollutants present in sewage including perylene and possibly oestrogenic compounds | Rose and Harshbarger, 1977 |

Bishop et al. (1991) found a strong statistical association between the presence of organochlorine contaminants (especially the PCB congener 2,3,3',4,4'-pentachlorobiphenyl) and decreased hatching success and increased developmental abnormalities in snapping turtles *(Chelydra serpentinia serpentinia*). However, the study could not demonstrate that any particular organochlorine chemical analyzed was the responsible agent.

In a field study with common snapping turtles in Ontario, Canada, the hypothesis was tested that snapping turtles from organochlorine contaminated sites would be feminized when compared to snapping turles from noncontaminated sites (De Solla et al., 1998). The authors found some evidence that the sexually dimorphic morphology of adult snapping turtles was more feminized at the contaminated sites. However no evidence was found that contaminants (PCBs/PCDDs/PCDFs and pesticides) had any effect upon testosterone or oestrogen levels. Sample sizes of testosterone measurements of males from some of the sites were small and therefore may not have been reliable. This study suggests that in adults external sexual development may be more sensitive to exposure to environmental contaminants than tesosterone production or metabolism.

One well documented case is that of the American alligator *(Alligator mississippiensis*) living in a contaminated lake in Florida (USA), Lake Apopka. The lake has contaminants derived from agricultural activities and municipal runoff and was also contaminated by a major spill of a pesticide mixure that was primarily composed of dicofol but also contained DDT in 1980 (see Crain et al., 1998). Alligator eggs from this lake contain elevated levels of a variety of persistent bioaccumulated pesticides and their metabolites such as *p,p'*-DDE, *p,p'*-DDD, trans-nonachlor, dieldrin, toxaphene,

oxychlordane, various PCBs and metals (Heinz et al., 1991). Significantly greater concentrations of *p,p'*-DDE have been found in fat and liver of juvenile alligators from Lake Apopka as compared to control lakes (see Guillette et al., 1996). The alligator population in Lake Apopka exhibited a 90% decline in juveniles during the years immediately following the spill. Alligator eggs obtained from Lake Apopka showed poor viability and poor offspring survival under controlled laboratory conditions (Woodward et al., 1993, Guillette et al., 1994). Developmental abnormalities in hatchling alligators included abnormal gonadal morphology, altered gonadal steroidogenesis, and abnormal sex steriod concentration in males and females (Guillette et al., 1994; 1995). Female alligators from Lake Apopka had abnormally high concentrations of 17β–oestradiol in blood plasma, for the 6 months after haching, whereas males had depressed testosterone levels and increased levels of 17β–oestradiol (Guilette et al., 1994). All neonatal females hatched from eggs collected on Lake Apopka had prominent polyovular follicles and many multinucleated oocytes, whereas polyovular follicles were not observed in controle females. Male juvenile alligators 6 months of age born after the spill had poorly organized testes with germ cells undergoing premature spermatogenesis and small phalli (Guillette et al., 1994). Thus, abnormal plasma steroid concentrations in the alligators of Lake Apopka correlated with altered gonadal morphology (Guillette et al., 1994) and smaller penis size (Guillette et al., 1996) in juvenile alligators.

To date the exact causitive agents of the endocrine disruption in Lake Apopka alligators has not been identified, although the principal contaminant found in alligator eggs and juveniles is *p,p'*-DDE. Many hypotheses have been proposed to explain the endocrine disrupting effects in male and female alligators. For example it is hypothesized that some of the endocrine disrupting effects ( such as polyovular follicles) are a reponse to embryonic exposure to EDC caused by the interaction of multiple EDCs with the alligator oestrogen receptor (Crain and Guillette, 1997). This is supported by the results obtained with traditional receptor competition assays which have shown that a number of the environmental oestrogens including those identified in the eggs of alligators living in Lake Apopka have the ability to bind to the alligator oestrogen and/or progesterone receptors (Vonier et al., 1996). Polyovular follicles in female alligators from Lake Apopka are very similar to those observed in mice treated with the estrogenic agent DES (see Bern, 1992). The observed developmental abnormalities in male and female alligators have also been linked to the anti-androgenic effects of *p,p'*-DDE. Androgens are essential for normal maturation and growth of the male reproductive system in reptiles (Guillette et al., 1996). It has been shown that *p,p'*-DDE displays anti-androgenic activity both *in vivo* and *in vitro* in rats (Kelce et al., 1995). In alligator eggs from Lake Apopka levels of *p,p'*-DDE (5.8 ppm wet weight) have been observed that are above the concentrations that block androgen receptor function *in vitro,* although very high doses were used to demonstrate antiandrogenic effects *in vivo* in rats (Kelce et al., 1995). It is therefore possible that this contaminant could act as an androgen antagonist in embryonic and juvenile alligators (Guillette et al., 1996). However to date there are no data available that indicate the ability of *p,p'*-DDE to bind the reptilian androgen receptor. It is, however, conceivable that multiple endocrine mechanisms including non-receptor mediated mechanisms are involved in the Apopka alligator case. *In vitro* studies suggested that the observed changes in reproductive and endocrine systems are the result of modifications in gonadal steroidogenic activity, and increased hepatic degradation of steroids (Guillette et al., 1995). Recent field and laboratory studies indicate that induction and suppression of aromatase enzyme activity are potential modes of contaminant-induced endocrine disruption in alligators (Crain et al. (1997). Recent field observations also suggest that the thyroid/gonad axis may be involved in the reported reproductive endocrine disruption (Crain et al., 1998). It is clear that further experimental and field studies are needed to provide causal relationships between pesticide exposure, especially during embryonic development, and the reproductive abnormalities in Lake Apopka alligators.

Laboratory studies have indicated that DDT may be oestrogenic in other reptilian and amphibian species. Palmer and Palmer (1995) reported that *o,p,*-DDT was able to induce the estrogen-regulated hepatic synthesis of the yolk-protein precursor, vitellogenin, in male red-eared turtles (*Trachemys scripta,*) and african clawed frog (*Xenopus laevis*). In a follow-up study by the same group (Palmer et al., 1997), vitellogenin induction in frogs was also demonstrated for dieldrin and toxaphene.

Amphibians have been mentioned by several bodies as an important group of wildlife species that deserve more attention. This class of vertebrates is subject to multiple exposures during different stages in their life cycle and therefore may be particularly at risk. It has been shown that known EDCs including several pesticides, can alter vitellogenin expression in a model amphibian species, *Xenopus* (Palmer and Palmer, 1995; Palmer et al., 1997; see also above). Further, thyroid hormones play a crucial role in the processes of metamorphosis and osmoregulation in amphibians (Galton, 1992; Shi, 1994) and fish (Norris, 1997) and the migrational behaviour driven by changes in buoyancy of amphibians (Reinke and Chedwick, 1939; Dent, 1985). Sofar, to our knowledge, no studies have been undertaken in Europe to investigate the possible effects of EDCc on wild amphibian populations. This is despite the fact that many amphibian populations are declining in both pristine and polluted habitats throughout the world (Blaustein and Wake, 1990; Carey and Bryant, 1995). Recently, increasing prevalences of amphibian deformities (e.g. malformed or lacking hindlimbs) in species in North America, central America and Australia have been reported (e.g. Burkhart et al., 1998). However, sofar the role of EDCs to the population declines or the deformity phenomenon remains unknown (Vial and Saylor, 1993). Apart from EDC effects in non-targeted amphibian species by pesticide spraying (Hall and Henry, 1992) few studies yet exist on whether reproduction of amphibian populations in the wild has been affected by environmental toxicants. The potential roles of environmental xenobiotics to decline of amphibian populations by impacting growth and development of the young have been examined by Carey and Bryant (1995). Their conclusion was that critical data are lacking in most cases. In another study, Rose and Harshbarger (1977) reported on inhibition of metamorphosis and high prevalences of skin lesions in the salamander *Ambystoma tigrinum* inhabiting a lagoon receiving secondary domestic sewage and perylene (a component of jet fuel). The authors suggested that the observed phenomena could have been caused by pollutants present in the sewage including oestrogenic compounds. The observation that the observed phenomena were reversed when animals were transferred to unpolluted ponds supported this hypothesis. A possible role of environmental factors including TPT and pH in growth and development of two toad species (*Rana lessonae* and *Rana esculenta*), in particular during the larval period, has been reported in a recent Swiss study (Fioramonti et al. ,1997). The two species have a sympatric and broad geographic distribution in Europe. The authors suggest that interspecies differences in these factors could have played a role in determining the variations of spatial distribution of these species and could have generally contributed to the decline of populations of sensitive amphibians. Recent studies have demonstrated that disturbances of the thyroid hormone system as a result of dosing amphibians with realistic levels of PCBs may result in distorted development of larvae, prolonged or even absent metamorphosis, or increased mortality depending on the life-stage at dosing (Gutleb et al., 1998). It is clear from the above that field and laboratory studies are necessary to determine the true scale of the problem, if any, in amphibian populations especially in Europe.

### 3.4 Fish

#### 3.4.1 Reproductive effects

Reproductive effects have been reported in various fish species in association with sewage effluent, paper industry and habitats (Table 7a).

## Table 7a: Reproductive effects in fish

| effect/ disorder | location | species | associated contaminants | reference |
|---|---|---|---|---|
| elevated blood vitellogenin in male fish | UK, estuaries semi-field study | rainbow trout *(Oncorhynchus mykiss* (caged) | sewage effluents: natural and synthetic oestrogenic hormones, nonylphenol | Harries et al., 1996, 1997 |
| elevated blood vitellogenin in male fish | Denmark semi-field study | rainbow trout *(Oncorhynchus mykiss* (caged | sewage effluents: natural and synthetic oestrogenic hormones, i.p. 17a-ethinyloestradiol | Larsson et al., 1999 |
| elevated vitellogenin in males, intersex (up to 100%) | UK rivers | roach (*Rutilus rutilus* | steroids | Purdom et al., 1994 ; Jobling et al., 1998; Routledge et al., 1998 |
| vitellogenin induction and reduced serum testosterone concen-trations in male fish | USA | carp *(Cyprinus carpio*) | metropolitan sewage treatment effluents | Folmar et al., 1996 |
| inhibition of spawning in female | United Kingdom | sand goby *(Pomato-schistus minutus*) | diluted sewage sludge | Waring et al., 1996 |
| elevated blood vitellogenin in male fish; ovotestis locally up to 20% | UK, estuaries and coastal waters | flounder (*Platichthys flesus*) | (xeno)oestrogens | Allen et al., 1999 Matthiessen et al., 1998 |
| elevated blood vitellogenin in female fish; testicular abnormalities | Scotland, estuaries | flounder (*Platichthys flesus*) | sewage and industrial waste: (xeno)oestrogens | Lye et al., 1997 |
| reduced plasma sex hormone levels, reduced gonad growth and delayed sexual maturity, | Sweden | several species | bleached kraft pulp mill effluents: chlorinated organic chemcials | Andersson et al. 1988; Sandström et al, 1997 |
| elevated blood vitellogenin in male fish | Sweden | perch *(Perca fluviatilis)* | pulp mill effluents: unknown chemicals | Förlin et al. 1999 |
| various reproductive, physiological and behavioural disturbances including masculinisation of females | USA, Florida | poecilid species including musquitofish (*Gambusia affinis affinis*) | pulp mill effluents: e.g. phytosterols (from trees) and organochlorines (PME) | Davis and Bartone, 1992; Drysdale and Bortone, 1989; Howell and Denton, 1989; Davis et al., 1992 |
| altered serum steroid levels | Lake Superior, Canada | white sucker (*Catastomus commersoni*) | e.g. bleached kraft mill effluent | Munkittrick et al., 1991; Van der Kraak et al., 1992 |
| delayed gonadal maturation, reduced egg size, increased fecundity | Canada, New Brunswick, Miramichi estuary | mummichog (*Fundulus heteroclitus)* | bleached kraft mill effluent | Leblanc et al., 1997 |
| reproductive impairment | Puget Sound, USA | English sole *(Parophrys vetulus)* | PCBs, PAHs | Johnson et al., 1988, 1997a,b |
| reproductive impairment | San Francisco bay, USA | starry flounder (*Platichthys stellatus*) | organic contaminants | Spies and Rice, 1988 |
| exposed males lead to decreased hatching succes of progeny | Canada, St Lawrence Bay, semi-field study | American plaice (*Hyppoglossoides platessoides*) | marine sediments contaminated with organic compounds | Nagler and Cyr, 1997 |
| decreased hatching succes | Baltic Sea | Baltic herring (*Clupea* | chlorinated hydrocarbons | Hansen et al., 1985 |

| | | | | |
|---|---|---|---|---|
| | | *hagengus*) | | |
| decreased fecundity | Baltic Sea | cod (*Gadus morhua*) | lipophilic xenobiotics | Peterson et al., 1997 |
| disruption of the normal ovarian cycle | France, coast of Britanny | plaice *(Pleuronectes platessa)* | Amoco Cadiz oil spill: petroleum contamination | Stott et al., 1983 |
| premature vitellogenesis (decreased turn-over of steroids) | Dutch, Texel semi-field study | flounder (*Platichthys flesus*) | harbour dredge contaminants | Janssen et al., 1997 |
| elevated blood vitellogenin in female fish | Boston Harbour, USA | winter flounder (*Pleuronectes americanus*) | unknown | Pereira et al., 1992 |
| decreased age at first maturation (1960- 1995) | North Sea | plaice, sole | unknown | Rijnsdorp and Vethaak, 1997 |
| changes in sex ratio (1981-1995) | North Sea | dab | unknown | Lang et al., 1995 |
| reproductive disorder M74 Syndrome (environmentally related disease 1974) | Baltic Sea | Atlantic salmon *(Salmo salar L.)* | dietary vitamin *B1* deficiency, but also chlorinated organic compounds, thyroid hormones and retinoids have been implicated | Akerman et al., in SEPA, 1995; Vuorinen et al., 1997a: Amcoff et al., 1998; Bengtsson et al., 1999 |
| reproductive disorder EMS Syndrome (Early Mortality Syndrome) | North American Great lakes | salmonids | dietary vitamin *B1* deficiency , but als chlorinated organic compounds have been implicated | Mac and Edsall,1991; Fitzsimons et al. 1999; AFS, 1996 |

Sewage effluent

Perhaps the best example of oestrogenic contamination in the aquatic environment is that resulting from the discharge of treated sewage to rivers. These discoveries have relied on the utility of the egg yolk precursor protein vitellogenin as a sensitive biomarker for oestrogen exposure in male fish. Treated domestic sewage discharges have been identified as a major cause of oestrogenic effects on fish in the UK and US fresh water environments (Purdom et al. 1994, Folmar et al. 1996). The UK studies are of particular relevance to the European situation and are discussed in detail in insert 4.

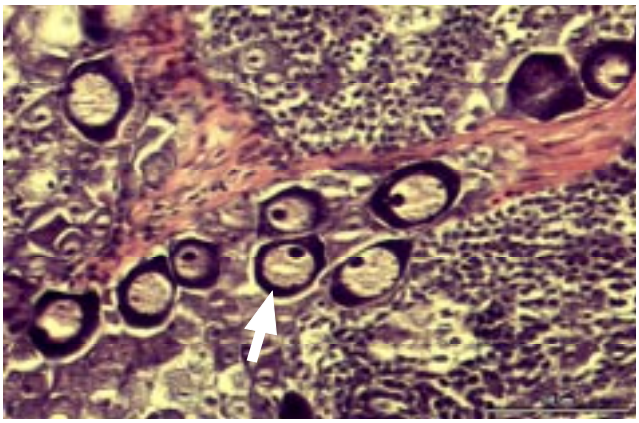
**INSERT 4**

**Oestrogenic effects in fish populations**

Studies in the UK have shown that a large number of sewage treatment plant effluents in this country are oestrogenic for fish (Purdom et al., 1994; Sumpter and Jobling, 1995) and that a number of receiving surface waters show oestrogenic activity (Harries et al., 1996). These studies revealed that caged male rainbow trout *(Oncorhynchus mykiss)* produce vitellogenin, and further also show a reduction in testicular growth. Although in first instance only alkylphenol derivates were identified as most likely causative agents (Jobling et al. 1996), later studies using bio-assay fractionation techniques showed that the oestrogenic activity of the water samples could also be explained by the presence of natural hormones (17β–oestradiol and oestrone) and to a lesser degree synthetic hormones deriving from contraconceptive agents (17α–ethinyloestradiol) (Desbrow et al., 1998). Recent surveys throughout the UK clearly demonstrated increased plasma levels of vitellogenin in wild populations of a freshwater fish, roach (*Rutilus rutilus)*, in a number of river systems that receive waste water effluents. Experimental findings indicate that environmentally relevant concentrations of natural steroidal oestrogens are sufficient to account for the levels of vitellogenesis observed in caged male fish placed downstream of certain sewage water treatment-effluent discharges in British rivers (Routledge et al., 1998). A large number of the examined roach from each of the surveyed rivers also showed a high prevalence (locally up to 100%) of intersexuality (ovotestis) (Jobling et al., 1998). These findings are proof of a widespread effect of oestrogenic compounds in wild fish populations in the United Kingdom. The observed reproductive disturbances are consistent with experimental findings and show an association with discharges of water treatment plants that are proven to contain oestrogenic steroids (Jobling et al., 1998). It appears, however, that low back-ground prevalence of ovotestis in roach can be found anywhere in the United Kingdom, but it is not know whether this is due to natural factors or to absence of completely pristine freshwater habitats. Whether the observed effects actually do have a negative impact on reproduction is still unknown. However, an experimental study reported by an other group in the United Kingdom has demonstrated inhibition of spawning in female sand goby (*Pomatoschistus minutus*) after exposure to diluted sewage sludge (Waring et al., 1996).

Another series of studies in the UK have concentrated on oestrogenic effects in the euryhaline flatfish flounder (*Platichthys flesus*) captured in the United Kingdom estuaries receiving municipal and industrial effluent (Allen et al., 1999). Flounder collected at inner stations on the Thames had moderately induced serum vitellogenin levels while outer stations had little induction. It was noted that flounder is less sensitive with respect to vitellogenin induction as compared to salmonid species. Similar moderate levels of induction were observed in the Clyde, while low or negligible levels of induction were observed in the Humber, Dee, Tamar, Alde and Crouch. High levels of induction were observed in the Tees estuary that receives inputs from several chemical manufacturers, and in particular elevated levels of nonylphenols. Similar high induction was seen in the Mersey and Tyne estuaries where nonylphenol inputs are thought to be smaller. In general, the data tentatively suggest that induction of flounder vitellogenin in the United Kingdom estuaries is not primarily related to the number of people in the catchment area but rather to the industrial effluent inputs and the chemical characteristics of those effluents. A graded response in vitellogenin induction was observed as samples were taken away from estuarine

sources. Oestrogenic effects could still be observed as slightly elevated levels at the offshore spawning areas in the Southern Bight (Allen et al. 1999). Induced vitellogenin persists in the blood of male fish for several weeks and it is therefore not unlikely that the origin of the exposure to oestrogenic compounds of the open sea spawning populations of flounder is largely from the open estuaries. However, the possibility of contamination in open sea can not be excluded.



**Histology of the testis of male flounder with characteristics of intersexuality (hermaphroditism): besides spermatogenesis also oocytes are present (arrow) (photo: SW Feist)**

Overall, the studies on wild male flounder have provided convincing evidence that oestrogenic exposure occurs in UK estuarine and marine waters at biologically significant concentrations, but preliminary studies of the possible effects indicate that intersex prevalence does not exceed 20% in fish from the most polluted estuaries. The causes of these phenomena in flounder are unknown but industrial effluents may play a greater role than seems to be the case in fresh waters (Matthiessen et al., 1998).

As shown in a recent paper by Larsson et al (1999), endocrine disruption may represent a widespread but largely unrecognized adverse health effect in fish downstream municipal sewage treatment works. Substances found include the synthetic oestrogen 17α–ethinyloestradiol, the natural oestrogens 17β–oestradiol and oestrone, as well as nonylphenol and bisphenol A. Following a two week exposure of caged male rainbow trout, elevated blood levels of vitellogenin were observed, and chemical analysis revealed that all the above mentioned oestrogenic compound were present in a conjugated form in the bile of these fish. The results were concluded to indicate that synthetic oestrogens (oral contraceptives) give a major contribution to the oestrogenic effect.

Laboratory experiments have shown that natural steroids and various xenobiotics are capable of inducing vitellogenin and the development of ovary tissue in male fish, but that interspecies differences clearly exist. For example, exposure of guppy (*Poecilia reticulata*) and medaka (*Oryzias latipes*) during 1 to 3 months to β-HCH caused particularly in guppy and to a lesser extent in medaka induction of vitellogenin, while ovotestis was only induced in medaka (Wester et al. 1985; Wester and Canton, 1986). Further, Gray and Metcalfe (1997) found intersex conditions in medaka exposed from hatch to 3 months of age: in fish exposed to 50 mg/l and 100 mg/l 4-nonylphenol, male fish developed 50 and 86% testis-ova, respectively. The ratio male/female for the 100 mg/l dose showed a significant altered sex ratio (1:2, M: F) in comparison with the controle (2:1, M:F). Young rainbow trout i.p. exposed to chlordecone, *o,p*-DDT and *o,p*-DDE showed *in vivo* induction of vitellogenin at relatively high liver residue concentrations, which indicates a lower oestrogenicity

of these compounds for this particular species (Donohoe & Curtis, 1996). Gimeno et al. (1996) showed that for carp (*Cyprinus carpio*) exposure to 17β-oestradiol, genetically male fish developed female sexual characteristics resulting in a total phenotypic sex reversal of the exposed fish. It is still not clear whether the observed effects in wildlife species such as the increased and non-functional vitellogenin production and ovotestis in males lead to adverse effects at the population level. The induction of vitellogenin in male rainbow trout has been associated with retarded testicular growth (Jobling et al. 1996). Further, it has been demonstrated that the unusual production of vitellogenin in male fish can result in kidney damage (Wester and Canton, 1986) and the suggestion is made that it may reduce metabolic expenditure on growth and spermatogenesis (Herman and Kincaid, 1988; see also Sheahan et al., 1994). An increased plasma level of vitellogenin has recently been associated - via a feedback mechanism - with reduced oestradiol production (Reis-Henriques et al. 1997), which in turn can negatively influence the egg quality. Vitellogenin induction in female fathead minnow (*Pimephales promelas*) exposed long-term (300 days) to 17α-ethynyloestradiol has been associated with an decreased egg production (Laenge et al., 1997).

Paper industry

The reproductive toxicity of bleached kraft pulp mill effluents (BKME) in fish was examined in a research program sponsored by the Swedish EPA. In the middle of 1980s fish caught near Swedish pulp mills frequently showed physiological disturbances including reduced plasma sex hormone levels, reduced gonad growth and delayed sexual maturity, suggesting exposure to endocrine disrupting substances (Andersson et al. 1988; Sandström et al, 1997). These effects were attributed to the chlorine bleaching process, which results in the formation and release of a vast number of chlorinated organic chemicals, many of unknown structure. As a result of these studies, the chlorine bleaching process was successively abandoned. Although reproductive disturbances and other fish health effects have been less frequently observed during the 1990s, reproductive disorders are still considered as a major remaining effect of pulp mill effluents. As determined by vitellogenin measurements in male and female perch *(Perca fluviatilis)* caught near pulp mills, a weak oestrogenic effect in male fish and possibly a weak anti-oestrogenic response in female fish have recently been indicated. The chemicals causing these effects are presently unknown (Förlin et al. 1999).

Endocrine disrupting of reproductive function resulting in masculination (changes of secondary sex characteristics) of fish from exposure to effluents from kraft pulp mills has been demonstrated in various studies in the USA and Canada (Munkittrick et al., 1991; Van der Kraak et al., 1992; McMaster et al., 1991; Gagnon et al., 1995; Leblanc et al., 1997). For example masculinization and a variety of other biochemical, physiological, metabolic, and behavioural disturbances were observed in viviparous poeciliid fishes during or after laboratory or *in situ* exposure to effluents from KME including a very complex mixture of natural and anthropogenic compounds (Drysdale and Bortone, 1989; Howell and Denton, 1989; Davis et al., 1992). Laboratory studies also showed lower egg production and delayed reproduction in fathead minnow exposed to BKME (Kovacs et al. 1995). *In vitro* studies of Timothy et al. (1995) demonstrate that pulp and paper mill black liquor and effluent fraction contain oestrogen- and dioxin-like compounds. Identified compounds include plant sterols, polychlorinated dibenzofurans and thianthrenes, dibenzothiophenes, and diphenyl sulfides, all of which have been detected in the vicinity of pulp and paper mills. Based on the dose-response curves, black liquor and the effluent fraction contain 10 ± 4 ppb and 20 ± 6 ppt "TCDD equivalents", respectively. Several studies have shown that TCDD and related compounds exhibit dioxin receptor-mediated anti-oestrogenic activity and can disrupt female reproduction in fish. In another field study, in white sucker (*Catostomus commersoni*), a large number of disturbances of the pituitary/hypothalamus axis at different levels was demonstrated as a consequence of exposure to BKME (Munkittrick et al. 1991; Van der Kraak et al. 1992), resulting in a disrupted steroid

synthesis by the ovary, a reduction of plasma steroid concentrations, and altered peripheral metabolism of steroids (Van der Kraak et al. 1992). The ecological significance of pollution induced sex alteration has been discussed by Davis et al. 1992. The combination of various studies and their own observations of the "highly" masculinised females from BKME effluents, let them to strongly suspect that reproductive function becomes impaired, if not entirely lost, after continued BKME exposure. Furthermore, the long-term effects of EDC would include reduced embryo viability and modification, or perhaps neutering of female reproductive function. More recently, Leblanc et al. (1997) observed a modification of the time of onset, intensity and duration of the spawning period in *Fundulus heteroclitus*, a small sentinel fish species, exposed to BKME in the Miramichi estuary, New Brunswick, Canada. The onset of gonadal maturation was delayed and egg size was reduced in fish captured downstream from the mill. However, at this site, fish also demonstrated a marked increase in reproductive investment and increased fecundity. A general problem when dealing with the complex BKME is the identification of the causative chemicals responsible for the observed reproductive effects.

Habitats

A number of field studies suggest a decreased reproductive success in fish as a consequence of exposure to lipophilic or yet unknown xenobiotics, including known endocrine disrupters in polluted habitats, such as industrialised estuaries and coastal waters (e.g. Johnson et al., 1988, 1997a,b; Spies and Rice, 1988; Nagler and Cyr, 1997; Hansen et al., 1985; Petersen et al., 1997). In most cases the ecological significance of these phenomena on reproductive output on the individual and populations level is not clear. Generally, knowledge on responsible mechanisms is lacking and no clear cause-and-effect relationships between specific chemicals and the reproductive effects observed have been established. For example, Johnson et al. (1988) described the inhibition of gonadal recrudescence and reduced levels of plasma oestradiol in female English sole *(Parophrys vetulus)* from a heavily polluted area. Since both conditions were associated with elevated hepatic aryl hydrocarbon hydroxylase activity, it was tentatively concluded that the disposition of oestradiol and consequently the ovarian development was influenced via interference of xenobiotics with enzymes involved in steroid metabolism. Of the two classes of xenobiotics suspected, aromatic hydrocarbons (AH) and organochlorine compounds, the AHs appeared to be most closely associated with the inhibited ovarian development and depressed plasma oestradiol. Johnson et al. (1997a) also reported, on an individual fish basis, elevated tissue PCB concentrations which were significantly correlated with low plasma alkali-labile protein (an indicator of vitellogenin levels), reduced egg weight, and reduced egg number, whereas elevated biliary fluorescent aromatic compounds were associated with increased ovarian atresia, increased egg weight and reduced egg number. The authors stated that nutrional and other environmental factors may also contribute to the observed intersite differences in egg weight and fecundity. In a more recent publication more evidence was found for a close association between aromatic hydrocarbons and inhibited gonadal development in adult sole, while both chlorinated (HCB, DDT and PCBs) and aromatic compounds were identified as potential risk factors for precocious maturation, a phenomenon which was not included in the previous studies (Johnson et al., 1997b). Histological examination of gonads obtained from plaice *(Pleuronectes platessa)* from petroleum contaminated estuaries following the Amoco Cadiz oil spill (1978) along the coast of Britanny showed evidence of disruption of the normal ovarian cycle, but the underlying mechanisms remain unclear (Stott et al., 1983). Laboratory studies show that a large variety of pollutants including heavy metals, pesticides and organochlorine pollutants may adversely affect reproduction in a large number of fish species and that exposure to 0.001 mg $l^{-1}$ (1 ppb) of pollutant is generally sufficient to produce harmful effects for long-term exposure (for review of literature up to 1993, see Kime, 1995). In a recent laboratory study, young rainbow trout exposed to environmental relevant concentrations of PCBs (Aroclor 1260) showed altered sex ratios (less females) and serious abnormalities of the gonads (inhibition of the development of the oocytes) (Baker Mata et al., 1998). It is not clear whether the observed phenomena are oestrogen receptor-

mediated or inhibition takes place of the enzymes that convert testosterone into oestrogen. The latter has been demonstrated in an other study in which short-term treatment with aromatase inhibitors resulted in masculinisation of female chinook salmon larvae (Piferrer et al. (1994).

Pollution-induced asynchrony in ovarian development (with 3 to 4 months) of flounder has been demonstrated in a 3 year semi-field study performed in The Netherlands. In this study, flounder were chronically exposed to polluted dredged spoil from Rotterdam harbour. Female fish exhibited premature vitellogenin production, which was attributed to enhanced plasma oestradiol. The authors also performed laboratory experiments and found indications that the increased oestradiol levels resulted from decreased clearance rather than enhanced ovarian production (Janssen et al., 1997). Pereira et al. (1992) have demonstrated enhanced blood vitellogenin in female winter flounder (*Pleuronectes americanus*) from polluted estuarine environments on the east coast of North America, although this also could be the result of impaired ovarian uptake of vitellogenin rather than elevated vitellogenin production.

Long-term data on fishery statistics were retrospectively analysed (1960-1995) to assess a possible effect of environmental contaminants on reproductive status and population size of commercial fish species (Rijnsdorp and Vethaak, 1997). The data on North Sea plaice (*Pleuronectes platessa*) and sole *(Solea solea)* populations showed a decrease in size and age to maturation, but no change in sex ratios were observed. Lang et al. (1995) have shown anomalies in the sex ratio of dab (*Limanda limanda*) from the North Sea, with increased representation of females in some areas, and decreased representation in others. In Canada, in a semi-field study, American plaice from the St. Lawrence have also shown a decrease in size and age to maturation (Nagler and Cyr, 1997). It appears unlikely, however, that these changes in North Sea plaice, sole and dab, and American plaice are significantly related to specific contaminants, but rather may be caused by changes in population dynamics.

M74 syndrome and EMS syndrome

An intriguing, but still unresolved case, is the M74 syndrome (environmentally related disease 1974) in Baltic salmon and the equivalent EMS (Early Mortality Syndrome) syndrome in salmonids of the North American Great lakes (AFS, 1996). The M74 syndrome has caused high mortality in fry of sea-run Atlantic salmon *(Salmo salar L.)* from the Baltic sea. The cause of M74 is largely unknown, but the pathology has been well described and treatment with thiamine (vitamin B1) in the hatcheries has been found to cure afflicted fry (Bengtsson et al., 1999). Reduced hatching succes and diminished fry survival has been observed in lake trout eggs experimentally exposed to PCBs and DDTs and it was found that there was a gradual increase in hatchability and fry survival of Lake Michigan trout since 1984 following a reduction in organochlorine levels in Great Lakes (Mac and Edsall (1991). The M74 syndrome shows great similarities to EMS in salmonids from the North American Great Lakes (Fitzsimons et al. 1999), a condition also characterized by a diet-related deficiency of thiamine in fry and broodstocks. A combination of factors may contribute to M74, e.g. the diet of salmon in the Baltic Sea, the content of thiamine or thiaminase in their prey fish, ecological changes in Baltic food chains, and environmental pollutants. One of the most probable hypothesis for these reproductive disorders is that toxic symptoms are expressed as a result of vitamin *B1* deficiency in the early life stages (Akerman et al., in SEPA, 1995; Amcoff et al., 1999). In a publication by Vuorinen et al. (1997a), dioxin-like compounds, especially certain PCDDs and PCDFs, were found to have a significant connection to the occurrence of M74 in Baltic salmon. However, there is no firm evidence to causally link these contaminants with the disease development. The same group (Vuorinen et al. 1997b) also suggested that changes in thyroid hormone status and retinoids may play a role in the M 74 syndrome, but the results in this respect are still inconclusive. A basic problem is to fully understand the environmental factors influencing the occurrence of this disease syndrome, which affects the few remaining wild, naturally spawning

populations of Baltic salmon that are at great risk of extinction. At present, there seems to be no firm evidence to support that endocrine-disrupting or other chemicals are involved in the aetiology of M74 and EMS.

### 3.4.2 Non-reproductive endocrine effects

Non-reproductive effects have been reported in several fish species (Table 7b).

**Table 7b: Non-reproductive effects in fish**

| effect/ disorder | location | species | associated contaminants | reference |
|---|---|---|---|---|
| embryonic malformation in pelagic eggs | North Sea coastal waters | various fish species | unknown | Cameron et al., 1992 |
| developmental defects in embryos | western Baltic Sea | various pelagic fish species | unknown | Westernhagen et al., 1988 |
| thyroid hyperplasia (up to 100%), decreased fecundity | Great Lakes, USA | salmonids | unknown | Leatherland, 1992 |
| reduced vitamin A levels | Dutch semi-field study | flounder (*Platichthys flesus*) | PHAHs | Besselink et al., 1998 |

Suggestions have been made that that larval deformities and increased mortality in pelagic eggs of various fish species and demersal eggs of herring in the Baltic Sea are caused by chlorinated hydrocarbons and other highly persistent bioaccumulative compounds (Von Westernhagen et al., 1988). In addition, there have been reports since the early 90s of a wide spread occurrence of embryonic deformities in pelagic eggs of several fish species in coastal waters in the North Sea (Cameron et al. 1992). It is not yet clear, however, whether these fluctuations reflect exposure to endocrine disrupters or other pollutants or may be caused by natural factors such as temperature, as the data also indicated a strong temperture-related correlation (Von Westernhagen and Dethlefsen, 1996).

Several studies have shown that potential adverse oestrogenic effects on fish can also alter growth in fish. Growth reduction has been observed in catfish exposed to DES (Bulkley, 1972). Johnstone et al. (1978) described a significant suppression of both length and weight in rainbow trout during a period of dietary administration of 17β-oestradiol. Of particular environmental concern are the recent experiments conducted by Ashfield et al. (1998). They examined in a series of experiments the long-term effects of different alkylphenol ethoxylates (nonylphenol, octylphenol, NP1EC) at environmentally relevant concentrations (1-50 µg/l) on growth and ovosomatic index in female juvenile rainbow trout. Exposure to alkylphenol ethoxylates commenced at hatch (day 0) to include the "critical window" of oestrogen sensitivity, and was terminated on day 22 or 35 followed by sampling at regular intervals up to 108 days and 466 days. From the results of both experiments it was apparent that all the test compounds modified the growth of exposed fish. They often found, however, variable physiological effects in the juvenile fish: no clear dose-related effect was observed. In most cases growth was suppressed, but in some cases growth was accelerated. The authors stated that very similar, rather variable effects on growth have been observed in juvenile rats exposed via their drinking water during pregnancy and lactation to alkylphenol compounds (Sharpe et al., 1995). The similarity between data obtained in the rat and fish study suggest that alkylphenol compounds are capable of influencing growth in both juvenile fish and mammals. Although it is possible that alkylphenol chemicals influence growth in fish and mammals via a direct oestrogenic

effect, other mechanisms of action are more likely. They concluded that exposure of natural populations of fish to these chemicals at concentrations currently measurable in the aquatic environment may have an impact on the performance of those populations. Survival of fish in the natural environment is strongly influenced by body size and an appropriate ovosomatic index is a crucial factor in successful reproduction. Survival of fish in the natural environment is closely linked to growth rate, primarily because smaller fish compete less successfully for resources, such as food and territory, than do larger fish (Elliot, 1990).

A large number of factors can influence the immune response in fish, including species, age, reproduction, social status, food, stress and water temperature (Anderson, 1993; Wester et al., 1994). Furthermore, several contaminants including endocrine disrupters such as PCBs, PCDDs, TBT may have a role in immunosuppression in fish which may be associated with an increased prevalence of diseases in wild populations (Vos et al., 1996). In the last decades there been have many reports indicating widespread occurrence of epizootic skin and liver diseases, including cancer, in wild fish populations in connection to polluted habitats (Vethaak and ap Rheinallt, 1992; Sindermann, 1993). However, there is absence of mechanistic and experimental data elucidating the possible effects of these compounds on fish disease.

Recent evidence demonstrates that oestrogens, either natural or xenobiotic, may promote growth of hepatic pre-neoplastic lesions and tumours in fish. Cooke and Hinton (1999) have shown that 17β-oestradiol is a tumour promotor in medaka and β-HCH a weakly positive modulator. 17β-oestradiol particularly promoted tumour development in male medaka, indicating xenobiotics with mechanisms of action like that of 17β-oestradiol may promote growth in wild fish of previously initiated cells into tumours (Cooke and Hinton, 1999). Although liver neoplasia in wild fish populations is primary attributed to the carcinogenic properties of polycyclic aromatic hydrocarbons (De Maagd and Vethaak, 1997, Myers et al., 1990), endocrine disrupting compounds could be involved as both risk factors for immunosuppression and tumour promotion.

Epizootics of thyroid hyperplasia and hypertrophy (affecting 100% of the population) have been reported in various species of salmon of the Great Lakes (see review of Leatherland, 1992). There is, however, no firm evidence linking thyroid hyperplasia observed in Great Lakes salmon with any specific chemical contamination (LC Formar, personal communication in EPA, 1997). Further, non-reproductive effects in fish include a significant reduction in retinoid levels in both liver and plasma in flounder in polluted mesocosms, providing a clear indication that retinoid levels are affected by long-term exposure to contaminants (Besselink et al., 1998). Together, these observations suggest that in fish, as in mammals, thyroid function appears to be sensitive to contaminant exposure.

### 3.5 Invertebrates

#### 3.5.1 Reproductive and non-reproductive effects

Reported studies of both reproductive and non-reproductive effects in various groups of invertebrates are considered in this paragraph (Tabel 8).

Although invertebrates constitute about 95% of the faunal species and are key components of all ecosystems, relatively little is know about their endocrine systems and their susceptibility to environmental endocrine disruption. Utilisation of hormones to control and coordinate biochemical, physiological and behavioural processes is common to all invertebrate taxa. The majority of invertebrate hormones identified to date are peptide neurohormones, but non-peptide endocrine messengers such as ecdysteroids and juvenile hormones are of importance in many groups especially insects and crustaceans (for review see Pinder and Pottinger, 1988). Both of these

systems are potentially susceptible to interference by environmental contaminants. A range of vertebrate steroids (androgens, oestrogens, progestins, corticosteroids) have been detected in insects and crustaceans, but their functional role remains to be established. Molluscs utilize a wide range of peptide hormones to controle and coordinate physiological processes, but ecdysteroids do not appear to play an important role in this group. The presence of vertebrate-type steroids has been reported for a number of molluscan species. In some cases, especially in the prosobranch gastropods, there is strong evidence that these steroids play a fucntional role. Evidence for a significant role of vertebrate-type steroids is strongest within the echinoderms where they may play a role in the control of growth and reproduction. For other invertebrate taxa there is only limited information on endocrine-type processes (Pinder and Pottinger, 1998). Given the physiological diversity it is likely that an endocrine disrupting chemical would have a different effect in different invertebrates.

To date, the tributyltin (TBT)-induced masculinisation (imposex/intersex) in female molluscs, particularly prosobranch snails belonging to the orders Mesogastropoda and Neogastropoda, is the best example of endocrine disruption in invertebrates that is causally linked to an environmental pollutant (Fioroni et al., 1991; Matthiessen and Gibbs, 1998). It is also a key example of population-level impact resulting from reproductive abnormalities. Wide-spread TBT contamination derives from the antifouling paint active ingredient TBT applied below the waterline on ship hulls. Imposex comprises the growth of a penis and occlusion of the oviduct due to the development of a superficial vas deferens. Whether imposex results in sterilization depends in part on the normal morphological configuration of the reproductive apparatus. For detailed information on TBT-induced imposex see insert 5.

**INSERT 5**

**Tributyltin-induced imposex in marine snail**

Imposex-affected populations have been observed in the wild in some 63 genera and 118 species (Fioroni et al., 1991) in marine prosobranch snails species from accross the world. Imposex-affected populations have been seen in Europe, in many estuaries (Gibbs et al. 1987; Huet et al., 1996; Fioroni et al., 1991; Oehlmann et al., 1996; 1998; Strand, 1998), the Iberian Atlantic and the Mediterranean Sea (Terlizzi et al., 1998; Morcillo and Porte, 1988; Sole et al., 1988; Coelho et al., 1998). All populations of the dogwhelk in the coastal areas of the North Sea are more or less affected by imposex leading in some areas to complete population loss (North Sea task Force, 1993). Signs of imposex were observed in the majority of neogastropod taxa in waters of British Columbia (Bright and Ellis, 1990). Recently, imposex has been reported in the common whelk *Buccinium undatum* from the open North Sea where 20 years ago no signs of imposex were observed, and this is apparently related to the intensity of commercial shipping traffic in the area resulting in release of the anti-fouling ingredient TBT (Ten Hallers-Tjabbes et al., 1994). Laboratory studies show that imposex development occurs at very low exposure concentrations to TBT (1 ng/l) in the dogwhelk and 7 ng/l in common whelk. The most sensitive species currently seems to be *Ocinebrina aciculata* with a treshold concentration of 0.1 ng TBT-Sn/l (see Oehlmann et al., 1996). Based on these laboratory studies with the wildlife species it is concluded that the concentrations of TBT measured in areas with imposex are sufficiently high to cause this condition.

**A female whelk from the open North Sea showing the development of a penis (imposex) (photo: H. Kralt)**

How TBT induces imposex is not precisely known, but a considerable weight of evidence suggests an inhibition of aromatase enzyme metabolism of testosterone to 17β–oestradiol (Bettin et al., 1996). This can lead to increased testosterone levels and masculinization. In the dogwhelk *(Nucella lapillis*, Neogastropoda) the degree of masculinization is a graded response dependent on the level TBT exposure. Imposex in the dogwhelk occurs at a TBT concentration as low as 1 ngSn.l (Oehlmann et al., 1993, Oehlmann and Bettin, 1996). Notably, exposure to testosterone may also lead to imposex. Simultaneous exposure to TBT (50 ngSn.l) and an anti-androgen (1.5 mg cyproterone acetate.l) did not result in imposex in contrast to the same concentration of TBT alone (Oehlmann et al., 1993). Exposure to the aromatase inhibitor SH-489 also resulted in imposex. These results can all be explained by the hypothesis that TBT interferes with the synthesis of steroid hormones by inhibiting the conversion of testosterone to oestradiol by the enzyme aromatase (CYP19). In addition to aromatase-inhibition an other hypothesis has recently been postulated, namely that TBT inhibits the formation of sulphate conjugates of testosterone, thus resulting in reduced elimination of testosterone from the body (Matthiessen and Gibbs, 1998). Furthermore, since TBT is reported to accumulate specifically in neural ganglia in the dogwhelk (Bryan et al., 1993) and common whelk (Mensink et al., 1997), it is possible that interference with the neuroendocrine system by TBT could result in the development of imposex, mediated by alteration of steroid metabolism. In England, Norway and in the Pacific Ocean recovery of imposex-affected populations of dogwhelks and whelks have occurred after measures were taken to cease the use of TBT on ships smaller than 25 meter (Tester al., 1996; Evans et al., 1994; Smith, 1996). In spite these reports that TBT concentrations in coastal waters and imposex occurrences in the UK and elsewhere are generally in decline, evidence put forward by other authors indicate that organotin concentrations are still high enough to endanger sensitive species (see Oehlmann et al., 1998).

Another condition termed intersex is observed exclusively in the periwinkle *(Littorina littorea*; Mesogastropoda). Intersex is defined as a disturbance of the phenotypic sex determination between gonad and genital tract and is characterized by supplantion of the distal part of the pallial oviduct by a prostate gland, a sperm groove and ultimately also a penis, whereas the proximal part of the pallial oviduct retains its female structure (Bauer et al., 1995; 1997). Intersex in the periwinkle occurs in populations in the German Wadden Sea (Bauer et al., 1995; 1997); the fertilty of female animals was affected in the direct vicinity of harbours and marinas. Intersex development was found at

concentrations of 10-15 ng/liter. In general juvenile animals are more sensitive for these effects than adult animals. For example, adult periwinkle are much less sensitive for TBT, although high concentrations show a significant decrease in egg production. In periwinkle, adult females loose their sensitivity to TBT once the whole pallial oviduct is established (Bauer et al., 1997). However, exposure to concentrations > 100 ng TBT/l reduces the egg production significantly in females of this species (Matthiessen et al., 1995). Laboratory experiments showed that exposure to concentrations higher than 10 ng TBT acetate/l induced the development of male sexual characteristics in a dose-dependent manner, when whelks were exposed *in ovo* or just after hatching. Growth rates were affected at an even lower dose. However, adult female whelks were not affected (Mensink et al., 1996).

In addition to TBT, antifouling paints may contain triphenyltin (TPhT), a chemical that is heavily used in agriculture as a fungicide, but has also been added to technical formulations of anti-fouling paints (up to 10% of the TBT concentration). TPhT has been found in sea stars and (imposex-affected populations of) whelks in the North Sea and Eastern Scheldt (NL) (Ariese et al., 1997; Mensink et al., 1997). Imposex was also found in Japanese waters in areas with a low shipping traffic intensity, but which are likely to receive TPhT compounds from neighboring agricultural araes (Horiguchi et al., 1994). Experimental evidence with regard to the ability to develop imposex due to TPhT exxposure is at present contradictory, since both negative and positive experimental studies have been reported (Bryan et al., 1988; Horiguchi et al., 1997). From the positive study it can be concluded that TPhT enhances imposex development in the rock shell (*Thais clavigera*). Thus, a role of TPhT with respect to the development of imposex seems quite likely in this species. Hawkins and Hutchinson (1990) also reported that monophenyltin (MPhT) induces imposex in *Ocenebra erinacea*.

**Table 8: Reproductive and non-reproductive effects in invertebrates**

| disorder/effect | location | species | associated contaminants | reference |
|---|---|---|---|---|
| imposex | world-wide different geografic regions | 140 species of prosobranchs and mesogastropods | TBT | Fioroni et al., 1991; Oehlmann et al., 1998 |
| imposex | UK and French coastal waters | dogwhelk *(Nucella lapillus*) | TBT | Gibbs et al. 1987; Bryan et al. 1988 Huet et al., 1996 |
| imposex | Danish waters | common whelk *(Buccinum undatum*) | TBT | Strand, 1998 |
| imposex | Iberian Atlantic Sea | several species of gastropods | TBT | Barreiro, 1998; Coelho et al., 1998; El Hamdani et al., 1998 |
| imposex and reduced growth | Eastern Scheldt, North Sea | common whelk *(Buccinum undatum)* | TBT | Mensink et al. 1997; Ten Hallers-Tjabbes et al. 1994 |
| imposex | Mediterranean | *Hexaplex trunculus, Bolinus brandaris* | TBT | Terlizzi et al., 1998; Morcillo and Porte, 1988; Sole et al., 1988 |
| masculinization (imposex) lose of reproductive potential | northeast Pacific | several neogastropod species | TBT | Bright and Ellis (1990) |
| imposex | Japanese waters | rock shell (*Thais* | TPhT | Horiguchi et al. 1994 |

| | | | | |
|---|---|---|---|---|
| | | *clavigera* | | |
| intersex | North Sea | periwinkle (*Littorina littorea*) | TBT | Bauer et al. 1995 |
| abnormally developed shells; declined spatfall | France, Arcachon Bay; UK and several other countries, estuaries | Pacific oyster *(Crassostrea gigas)* | TBT | Alzieu et al., 1986, Alzieu et al., 1991; Waldock and Thain, 1983; Stephenson, 1991 |
| abnormal sex ratios | experimental study, Scotland | harpacticoid copepods | sewage | Moore and Stevenson, 1994 |
| previtellogenic oocytes; altered steroid profiles in testes and hemolymph | Atlantic Canada, Halifax Harbor | lobster (*Homarus americanus*) | contaminants | Sangalang and Jones, 1997 |
| effects on CYP450 monooxygenase; no effect on oocyte maturation | North Sea | sea star (*Asteria rubens*) | pollutants unknown | Postma and Valk, 1996 |

In several species of bivalve molluscs, increased shell thickness resulting from TBT exposure has been reported. In bivalves, TBT is highly toxic to larvae and gives rise to malformations of the shells of adult animals by inhibition of calcification. Changes in morphology of the sexual organs have not been found in these species (Alzieu et al., 1991).

Deleterious effects of organotin compounds were first noted in Pacific oyster *(Crassostrea gigas)* populations in Arcachon Bay, France in the late 1970s: from 1977 to 1983 oyster production was severely affected (Alzieu et al., 1986; Alzieu et al., 1991). Shells developed abnormally and spatfall declined dramatically. Abnormally developed shells have been observed also in the UK and in estuaries of several other countries (Waldock and Thain, 1983; Stephenson, 1991**).** Shell abnormalities included chambering and the formation of a protein-containing jelly and effects on calcification occurred at concentrations of TBT as low as 2 ng/l although the sensitivity varied between oyster species. Larvae survived only a few days in Arcachon Bay water and retarded growth and high mortality occurred in larvae exposed to 50 ng/l of TBT in laboratory tests. At a concentration of 20 ng/l growth of *C. gigas* was inhibited but the related species *C. virginica* was less sensitive. Effects of TBT on reproduction have also been reported for several other bivalve molluscs including the native oyster *Ostrea edulis* (Thain et al., 1986) and the deposit feeding clam *Scrobicularia plana* (Ruiz et al., 1994; 1995a,b). In the latter field and laboratory studies it was found that the toxic action of TBT has a negative influence on embryonic development and could have contributed in northern Europe to population declines or disapparences from certain estuarine sites. Remarkably, there are no reports in the literature on effects of TBT and related compounds in fresh water molluscs. From a population point of view the effects on reproduction are obviously more important than shell deformities (see above). The underlying mechanism by which TBT causes shell thickening in Pacific oysters is presently unknown.

There is evidence that the endocrine disrupting effects of TBT have led to changes in marine communities. Detailed temporal trend studies of benthic and epibenthic community structure (Waldock et al., 1999; Rees et al., 1999) have been conducted in the Crouch estuary, UK, since 1987 when the use of TBT for small vessels was banned. The estuary is rather unique because of its primary TBT inputs; other polluting inputs to the estuary have been negligable historically. The findings show over a period of 5 to 10 years a considerable increase in species diversity in the upper- and mid-estuarine areas where TBT concentrations declined to relatively low levels. However, there were no significant changes in the originally less-contaminated areas of the outer estuary. It is therefore likely that the effects were at least partly due to the decline in TBT input. Recovery was seen not only in several mollusc species (including the oyster *Ostrea edulis*), but also

in crustaceans and ascidians, although it is not known whether these latter groups were originally affected directly or indirectly.

Another observation in invertebrates concerns the very high proportion of intersex in hapacticoid copepods in the Firth of Forth in Scotland (Moore and Stevenson, 1994). This observation has been linked but not confirmed to a sewage effluent with elevated levels of alkylphenoxy-ethalates from the local textile industry. Furthermore, the lobster *Homarus americanus* has been observed with ovotestis in Nova Scotia but it was not clear whether this was a natural or site-related phenomenon (Sangalang and Jones, 1997).

Semi-field studies with the echinoderm *Asterias rubens* have shown that rates of P450-dependent steroid metabolism can be decreased as a result of exposure to contaminants. Short-term cadmium exposure of this animal affected steroid synthesis and tissue levels of progesterone and testosterone (Voogt et al. 1987). Den Besten (1991a,b) found that both cadmium and PCBs significantly reduced levels of progesterone and testosterone in the pyloric caeca of male and female *Asterias rubens*. No effect of cadmium was found in the gonads, while PCB exposure resulted in increased testosterone levels in testes and ovaries. Although the physiological role of progesterone and testosterone in sea stars is still unknown, it has been suggested that changes in their levels represent a hormonal trigger for the pyloric caeca, being the onset of vitellogenesis and gametogenesis (Voogt et al., 1987). Results from field studies are inconclusive. In a number of surveys in the North Sea, *Asteria rubens* were collected at different sites in order to investigate the quality of their offspring and to perform biomarker measurements. No pollution gradient-related effects were observed on oocyte maturation or on the early development of sea star embryos. However slight effects were observed on the P450 system in the pyloric caeca of female sea stars collected from polluted coastal sites under the influence of rivers like Humber, Elbe, Rhine and Western Scheldt (Postma and Valk, 1996). Given the experimental findings, these decreased P450 activities found in the North Sea study may signal adverse effects.

A range of pesticides that act on insect endocrine systems have been deliberately introduced in the environment and have shown to have also impacts on non-target species. There are no documented examples of non-pesticidial EDC-related effects on freshwater and terrestrial invertebrates. There are, however, a substantial number of laboratory based studies which identify the effects of potential EDCs on freshwater and terrestrial invertebrate species (for literature overview see Pinder and Pottinger, 1998; Janssen et al., 1998). Woin and Bronmark (1992) showed that exposure to non-lethal concentrations of DDT cause a substantial reduction in the fecundity of the freshwater snail *(Lymnaea stagnalis).* More than 100 phytosteroids are structurally similar to ecdysteroids and have been shown to exert hormonal activity in insects (Adler and Grebenok, 1996). For example, moulting was arrested in dargonfly larvae (*Macromia cingulata*) when exposed to paper and pulp mill effluent containing phytoecdysteroids (Subramanian and Varadaraj, 1993). Alkylphenol (4-NP) apparently reduces the rate of elimination of testosterone in freshwater crustacean *Daphnia magna* leading to accumulation of androgenic products and reduced fecundity of females (Baldwin et al., 1997). The alkylphenol p-*tert*-pentylphenol (PTP) may induce irreversible changes in morphology and reproduction of *Daphnia magna* including masculinization of females (Gerritsen, 1997).

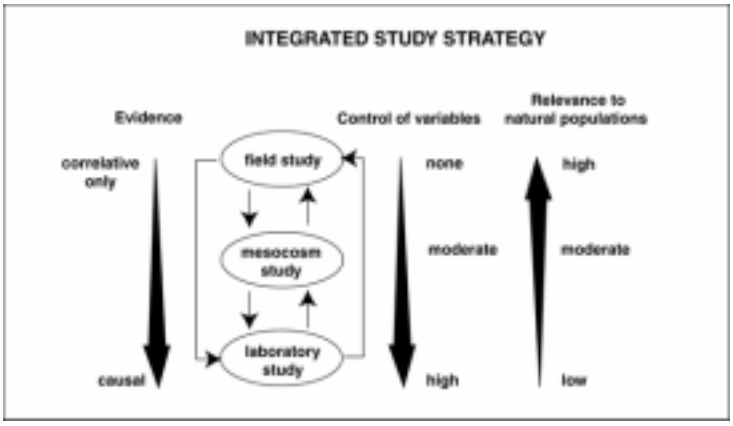
### 3.6 Weight of evidence on wildlife health effects

The precise endocrine mechanisms regulating the reproductive process of a species are diverse and may be influenced by a range of biological and physico-chemical factors. These environmental factors include xeno-oestrogens and other chemicals observed to affect the reproductive physiology of fish and other wildlife species (Campbell and Hutchinson, 1998). It is also recognized that environmental stress can have an effect on the survival and immunocompetence of fish populations

and that such stress can reduce the reproductive success of aquatic and terrestrial species. In addition nutritional deficiencies may have profound effects on the survival, development and reproductive health of organisms. Therefore there is no doubt that a variety of factors impinge upon the reproductive output and fitness of natural populations. Many feedback mechanisms and redundancies at various levels of organization have to be overruled before populations are affected.

As follows from the definition adopted by the working group, an endocrine disrupter can only be defined in a complete organism containing a functional endocrine system. The ecological significance of such a compound needs to be demonstrated in a free-living animal exposed in its natural habitat. Consequently, a full understanding of a health effect resulting from endocrine disruption in a wild population requires a combination of experimental, mechanistic studies under controlled exposure conditions and an association between a health effect and exposure to a defined chemical or group of chemicals in the real world.

The ultimate goal of ecological risk assessment is to protect communities and whole ecosytems from detrimental effects caused by chemical pollutants (Landis et al., 1995). To date, however, there seems to be no good example to demonstrate that a defined toxic effect resulting from exposure to an endocrine disrupter has caused an adverse effect that becomes visible at the ecosystem or community level, with the exception of community effects of TBT. Moreover, as is evident from the overview of data given above, there are only a few cases where an observed ecotoxicological effect on the population level can be firmly linked to a chemical acting on the endocrine system. Some of these cases have been highlighted above. This situation is not unique for endocrine disrupter research but is rather a situation characteristic for (eco)epidemiology in general. While the ecoepidemiological approach aims at establishing relationships between reproductive and health disturbances and exposure to environmental pollutants in wild populations, it will generally provide little information about the underlying mechanisms of action of the chemicals involved. It is therefore evident that an evaluation of the ecological significance of endocrine disrupting chemicals needs to be based on a combination of ecoepidemiological field studies and controlled experimental studies. The strengths and weaknesses of these approaches are depicted in the figure below.




**Integrated approach to study the causes of wildlife diseases (from: Vethaak, 1993) (”low relevance to natural populations=needs interpretation”; “mesocosm study=semi-field”)**

As indicated in the figure, the so called semi-field studies would represent a useful approach to bridge the gap between the controlled conditions of the laboratory experiments and the uncontrolled exposure conditions in the field. The use of sentinel species to examine markers for exposure and

biological effects, and the application of defined test organisms to determine effects of exposure at contaminated sites represent promising approaches of semi-field type (*in situ* experiments).

While it seems difficult to reach definite conclusions about cause-effect relationships in the field, there are numerous examples of ecotoxicological effects where a mechanism of endocrine disruption would seem possible or even likely. Several of these cases refer to persistent organic pollutants, many of which are known to exert experimental toxicity by different mechanisms of action relating to endocrine disruption. Compounds of this type include chlorinated hydrocarbons such as PCBs, PCDFs and PCDDs, as well as hydroxylated and methylsulphonylated metabolites of PCBs and DDT.

The best documented cases of reproductive disturbances and population declines due to exposure by EDCs in Europe and abroad include: TBT-induced imposex in marine snails, DDE-induced eggshell thinning in birds, and reproductive and immunosuppressive effects in seals by PCBs and metabolites. It is remarkable, that in the discussed cases of EDC exposure, notably in roach, despite widespread vitellogenin induction and ovotestis formation no effect has been reported at the population level. For most studies included in this overview, however, the evidence for a causal link to endocrine disruption is weak or non-existing. This may be due to the complexity of contaminant mixtures, the lack of chemical exposure data, of data on the sensitivity of the species concerned, and of knowledge on mechanisms of action. Specific remarks for the various animal groups are given below.

Mammals and birds: Most studies on endocrine modulating effects in mammals refer to aquatic mammalian species, only few to terrestrial species. In most cases the causal evidence for effects due to EDC in terrestrial and aquatic mammals remains circumstantial. Apart from the PCB-induced decline of the grey seal population in the Baltic Sea (see above), probably the best evidence for cause-effect relationships comes from Dutch semi-field studies in harbour seals, in which both reproduction and immune function have been impaired, and PCB have been implicated. In some cases good correlations are found between on the one hand concentrations of certain organochlorines and their metabolites and on the other hand hormone concentrations and observed disorders, but the mode of action is not very well understood. In many other cases no clear-cut correlations between organochlorine-residue levels and observed effects could be established. This is mainly due to the incomplete nature of the data, e.g. lack of data on residue levels, and lack of toxicological (dose-response relationships) and ecological background data such as normal population parameter values. The complexity of a causal relationship is illustrated by the observation that some of the affected populations to date are growing in number despite clear localised evidence of effects caused by exposure to EDC. An example is the recently increased population of the Baltic grey seals, which occurred despite the fact that this population locally still suffers from pathological lesions induced by persistent organochlorines.

Reptiles/amphibians: There is increasing evidence from non-European countries that some chlorinated aromatic compounds are capable of disrupting reproductive, developmental and behavioural functions in reptiles and amphibians. Experimental data indicate that larval stages of amphibians may be particularly sensitive to endocrine disruption. So far no effects on amphibian and reptile populations in Europe have been demonstrated**,** possibly because such investigations have as yet not been conducted. Therefore field and semi-field studies are needed throughout Europe to examine the population status of amphibian**s** and reptiles, and the possible impact of EDC on these populations.

Fish: There is convincing evidence that the endocrine system of a variety of fish species has been adversely affected by endocrine disrupting chemicals. The early data indicate that effects occur at

geographically defined contaminated sites and sources (e.g. effluents of water treatment plants, wood- and paper processing industries, textile industry). This exposure appears to be associated with endocrine disruption and reduced reproductive success which could result in decreased fish populations. However, there are recent indications for a more widespread occurrence of oestrogenic effects in certain fish populations in the United Kingdom. Significant oestrogenic exposure predominantly due to natural and synthetic steroids but also to other compounds is not restricted to freshwater systems, but is also demonstrated in estuaries, fjords and sheltered coastal areas. Endocrine disrupting compounds associated with physiological and reproductive effects in fish to date are natural and synthetic as well as plant-derived steroids. Oestrogenic effects observed in fish in industrialized estuaries in the North Sea and elsewhere, however, indicate that industrial effluents may play a more important role**,** including alkyl phenols, and conventional problem substances such as DDT, PCBs/PCDFs/PCDDs, heavy metals as well as yet unidentified chemicals. It is unclear to what extent endocrine disrupting chemicals are implicated in the decline of fresh water/coastal fish populations**,** although natural factors such as habitat destruction and fisheries impact may override the contaminant effects in most instances. Effects on important offshore fish stocks have not been demonstrated so far, although certain North Sea flatfish appear to be maturing earlier than 30 years ago. However, this effect could be explained by a natural cause. In most cases the biological and ecological significance of observed changes such as precocious female maturation, intersexuality, ovotestis formation, vitellogenin induction for reproductive success is unclear and merits further examination.

Little is known about non-reproductive endocrine effects of contaminants in fish, although an endocrine disrupting component could play a role in embryo malformation, immunosuppression, decreased vitamin A levels, thiamine deficiency, infectious disease, and epizootic liver tumours in wild fish populations. Chemically-induced alterations in immune function occurring in early life-stages or in adult fish can translate into an altered host resistance and susceptibility to infectious disease (Wingspread, 1996). Clearly, more research is required to understand the role of contaminants in susceptibility of fish species to infectious diseases. In particular, more emphasis should be placed on immunosuppressive effects of TBT.

Shellfish and other invertebrates: Perhaps the best example of an endocrine disruption effect is that of TBT causing imposex worldwide in many species of marine snails. This phenomenon is held responsible for global declines in populations of several species. There is now an understanding of the effects at all levels of biological organisation from molecular to population levels. TBT is considered to inhibit the cytochrome P450-dependent aromatase (CYP 19) activity responsible for conversion of testosterone to oestradiol. Apart from the example of imposex in marine snails, EDC-induced effects in other invertebrates are poorly documented. Not surprisingly, effects of pesticidal endocrine disruptors on development of single organisms or populations of non-target species have been identified. However, it is likely that endocrine disruption is also occurring in other invertebrates (e.g. crustaceans). Appropriate field studies are therefore needed to investigate the effects of EDCs on invertebrates, especially in the fresh water environment.

## 4. EU testing strategies for chemicals, including exposure considerations

For specific groups of chemicals such as pharmaceuticals and pesticides routine testing for a wide range of biological effects has long been required at a national level. These requirements reflect the obvious concern for the effects of substances deliberately developed for their biological activity. More recently, harmonised requirements for testing have been introduced at EU level.

For pesticides, the testing requirements are given in Annexes II and III to Directive 91/414/EEC. (EEC, 1991, EC 1994b, EC 1996a). Testing of these plant protection products is required both for the active substance (Annex II) and for the diferent formulations containing the substance (Annex III). The tests required for both human health and the environment are considerable (see Tables 9a and b).

**Table 9a: Toxicological and metabolism studies on a plant protection product active substance**

| | |
|---|---|
| **toxicokinetics** | studies on absorption, distribution, excretion and metabolism in mammals |
| **acute toxicity** | oral, percutaneous, inhalation |
| **irritation** | skin and eye irritation |
| **sensitisation** | skin sensitization |
| **short-term toxicity** | oral 28-day toxicity study<br>oral 90-day toxicity study<br>other routes (inhalation, percutaneous as appropriate)<br>delayed neurotoxicity studies: OECD Guideline 418 |
| **genotoxicity testing** | *in vitro* studies: *Salmonella typhimurium* reverse mutation assay, mammalian cytogenetic test, mammalian cell gene mutation test<br>*in vivo* studies in somatic cells: micronucleus test, mouse spot test, mammalian bone-marrow cytogenetic test, chromosomal analysis.<br>*in vivo* studies in germ cells: on a case by case basis. Suitable tests would need to examine interaction with DNA (such as the dominant lethal assay), to look at the potential for inherited effects and possibly make a quantitative assessment of heritable effects. |
| **long-term toxicity and carcinogenicity** | chronic toxicity test, carcinogenicity test or combined chronic toxicity/carcinogenicity test. |
| **Reproductive toxicity** | two-generation reproduction toxicity test (and supplementary studies), developmental toxicity studies, teratogenicity test - rodent and non-rodent. |
| **Other toxicological studies** | toxicity studies of metabolites. In certain cases it can be necessary to carry out supplementary studies to further clarify observed effects. These studies could include:<br>- studies on absorption, distribution, excretion and metabolism,<br>- studies on the neurotoxic potential,<br>- studies on the immunotoxicological potential,<br>- studies on other routes of administration. |
| **Medical data** | medical surveillance on manufacturing plant personnel<br>direct observation, e.g. clinical cases and poisoning incidents<br>health records, both from industry and agriculture<br>observations on exposure of the general population and epidemiological studies |

The data requirements of this Directive should provide sufficient information to determine any possible endocrine disrupting activity in mammals when enhanced 407 and 416 tests are used.

Regarding ecotoxicological assessment, the studies required on the actiove substance are also extensive.

**Table 9b: Ecotoxicological studies on a plant protection product active substance**

| | |
|---|---|
| **birds** | acute oral toxicity, short-term (OECD 206) |
| **aquatic organisms** | acute toxicity to fish, chronic toxicity to fish, fish early life stage toxicity test (OECD 210), bioconcentration in fish (OECD 305E), acute toxicity to aquatic invertebrates, chronic toxicity to aquatic invertebrates (OECD 202, Part II), effects on algal growth, effects on sediment dwelling organisms, aquatic plants |
| **arthropods** | bees: acute toxicity (EPPO guideline 170), bee brood feeding test (ICPBR method)<br>other arethropods: two sensitive standard species, a parasitoid and predatory mite should be tested. |
| **Earthworms** | acute toxicity, sublethal effects, |
| **soil non-target micro-organisms** | |
| **other non-target organisms (flora and fauna)** | |

These tests on the active substance are done on the basis of laboratory tests. The results of these tests may in the case of chronic studies in the aquatic environment give relevant information. However, the requirements for studies on the formulation include multispecies designs and information at the population/community levels using mesocosms and/or field studies. For formulations of plant protection products tested at mecocosm or field study level, it must be concluded that any ecologically relevant effect, including those exclusively or non-exclusively related to endocrine disruption, will be detected and therefore assessed at this level, even if the mode of action is not identified.

In addition, data from human toxicology testing can be applied to the environment as well. The chronic toxicity tests on mammals are used for both human health risk assessment and ecological risk assessment. In order to do this, two different NO(A)EL values are calculated for each test, one is the classical value for toxicologically relevant effects, the other is the ecologically relevant NO(A)EL value representing a toxicity endpoint which can affect the population response.

The systematic testing of industrial chemicals was introduced in the European Community with the 6th. Amendment to Council Directive 67/548/EEC (EEC, 1979). The background for the discussion was that unlike pesticides, there was no systematic requirements for testing industrial chemicals in general. The numbers of chemicals involved are large, and, at the time, there was no reliable inventory of chemicals on the market. The agreement reached at this time was that it was not realistic to introduce a general testing requirement for all industrial chemicals, but that testing requirements should be introduced for any new substances not previously put on the market.

In order to provide a basis on which it was possible to distinguish substances already on the market from new substances, the European Inventory of Existing Commercial Substances (the EINECS

Inventory) was compiled. This list includes slightly over 100,000 substances reported to be on the European market in the ten-year period prior to 18 September, 1981 (EEC, 1990).

The 6th Amendment introduced testing requirements for substances not included in this inventory. The main intention of the testing requirements was to ensure that a so-called “base-set” of information was collected (Annex VII A to the Directive, EEC 1992a).

This “base-set” of data includes information on the manufacturer or importer and information on the identity of the substance. Information on the use of the substance is also required, including details of production, proposed uses, estimated production, recommended methods for handling, storage, etc., emergency measures and packing requirements. Data also has to be provided on a range of physical-chemical properties.

There are also requirements for a range of toxicological and ecotoxicological properties. Substances must be tested for acute toxicity by inhalation (for gases and volatile liquids) and by oral administration and by either dermal or inhalational administration as appropriate for other non-volatile substances. Skin and eye irritation and skin sensitisation must also be tested. For repeated toxicity, a 28-day study by an appropriate route (usually oral) is required, and mutagenicity must be studied in two tests (one a bacterial test, with and without metabolic activation; the second should be a non-bacteriological test to detect chromosomal aberrations or damage). A screening test for reproductive toxicity is required, but no such test has yet been included as an agreed test method in the Directive. Also, an assessment of the toxicokinetic behaviour of the substance can be required.

Finally a number of tests for effects on the (largely aquatic) environment are required. These include acute toxicity studies in fish, daphnia and growth inhibition test on algae; a measure of bacterial inhibition (in cases where this may affect biodegradation) and tests for biotic and abiotic degradation.

The tests selected give a good basis on which to judge a number of aspects of the biological activity of the substance, but do not by any means cover all possible end-points of potential interest. In particular there is only limited information available from these tests on effects such as cancer and reproductive toxicity.

The Directive includes modifications to this base set. For substances produced in volumes of less than 1 tonne per year in the European Union, there are reductions in the amount of tests needed to be carried out. For substances produced in quantities between 100 kg and 1 tonne per manufacturer, a reduced base set is required where the full base set of toxicological studies is required with the exception of a repeated dose study (28 days), non-bacterial mutagenicity studies, screening for reproductive toxicity and the toxicokokinetic assessment. Testing for the environment is limited to measurement of biotic degradation (Annex VIIB, EEC 1992a). If a substance is produced in quantities of less than 100 kg per year per manufacturer, the list is considerably reduced. For toxicity studies, only data on the acute toxicity is required and no test for effects on reproduction (Annex VII C, EEC 1992a).

For substances produced in larger volumes, the base set is extended (Appendix VIII A, EEC 1992a). For substances marketed in quantities over 10 tonnes per year per manufacturer (or 50 tonnes per year in total in the Community) additional tests are necessary. Additional toxicity studies include fertility studies (one species, one generation), and, where positive results have been seen, a second generation should be studied. Teratology must be studied if this has not been studied in the fertility study. Sub-chronic (90 day studies) are normally required and additional mutagenicity studies are normally required at this stage. In addition basic toxicokinetic information needs to be supplied. A

series of additional tests are required for the environment, included chronic studies for the aquatic environment, and tests on earthworms and plants.

These tests may be required by the national Competent Authorities if they feel that there is a justification to do so when the quantities of the substance produced by a single manufacturer exceed 10 tonnes per year. However; when the quantities exceed 100 tonnes per year the "burden of proof" is reversed: the Competent Authorities are required to request this information, unless the producer can provide good reasons why these studies should not be carried out.

For new notified substances produced in even larger volumes, (1000 tonnes per year), a range of additional studies can be required. These include a chronic toxicity study, a carcinogenicity study, a fertility study (e.g. three generation), developmental study (including peri- and post-natal effects), a teratology study (in a second species), additional toxicokinetic studies, and studies to investigate organ or system toxicity.

In introducing these requirements based on production volume, the Directive makes an implicit use of Risk Assessment concepts. The production volume is used as a surrogate for exposure, and the testing strategy adjusted to this. This is clearly not a justified assumption in all cases. As a result, the 7th. Amendment to the Directive introduced the requirement to carry out a Risk Assessment for the new notified substances. In order to carry out this Risk Assessment, a Commission Directive (EEC 1993c) was adopted which sets out the general basis for the assessment. This Directive does not attempt to include the many technical considerations that are required for carrying out this procedure. Detailed advice has been published in the form of a Technical Guidance document (EC, 1996c, 1997a). This includes detailed comments and advice on possible testing strategies to investigate different toxicological concerns.

The system therefore when risk assessment and testing strategies are included provides the opportunity for an increasing body of experimental data to be acquired as production volume increases. Normally, where no special issues of concern are involved, the tests carried out will in general follow the production volumes described above.

The testing strategies described in the Technical Guidance documents, together with the application of the results of a risk assessment can be used as the basis to require additional testing at an earlier time than production volume considerations would normally require. However, if additional testing is required, then this must be based on clear justifications, for example on *in vitro* or *in vivo* prescreening results or structure activity relationships.

For the existing substances listed in EINECS, systematic testing requirements were first introduced in 1993 (EEC 1993a). The very large numbers of substances involved have led to the introduction of phased requirements. The first phase requires that all available existing information is supplied to the Commission. For substances placed on the European market in amounts greater than 10 tonnes per year, the information is required to be sent to the Commission by 28 June 1998.

Secondly, a number of substances are nominated on one of three priority lists (EC, 1994a, 1995, 1997c). A risk assessment for each of these substances is carried out by a Rapporteur (a member state). For these substances, data must be available to cover all the requirements listed in the base set for new notified chemicals. In addition, additional tests may be required by the Rapporteur.

Finally, there are provisions in the Regulation that enable the Commission to require specific testing of existing substances "where there are valid reasons for believing that a substance appearing in EINECS may present a serious risk to man or to the environment, a decision to ask the

manufacturer(s) and importer(s) of the said substance to supply the information which they possess and/or to subject the existing substance to testing and to provide a report thereon". These requirements take the form of a Commission Regulation which is subject to vote in a Committee procedure. This provision has been used to acquire exposure data on a number of substances (EC 1997b).

Inclusion of tests for concerns not originally addressed by the base set can therefore be dealt with in a number of ways.

Additional tests for new chemicals could be included in the different base set test requirements at various production levels. This could be done by the introduction of appropriate test methods in Annex V to the Directive (EEC 1988, EEC 1992b, EEC 1993b, EC 1996b, EC 1998), and the inclusion of references to these tests in the appropriate annexes (VII or VIII).

Alternatively, particular concerns (associated with chemical structure, or prompted by the result of other studies, already included in the base sets) could be addressed by inclusion of appropriate advice in the testing strategies included in the Technical Guidance for carrying out a risk assessment.

As the current OECD 407 protocol serves as a basis for detection of adverse effects on the endocrine system relevant for humans there is an obvious need to put more emphasis on adverse effects on the endocrine system from the 28 day study in rats. This could be done by enhancements of parameters as potentially useful for detection of effects on the endocrine system (CSTEE/98/9 – Add 13).

Finally, for existing chemicals, the possibility exists to require specific testing for endocrine disrupters on the basis of a Commission Regulation.

## 5. The utility of ecotoxicity testing for ecosystem protection

The previous section has presented an overview of the current EU testing strategy for chemical substances. For the environment, both, the hazard identification and the effect evaluation part of risk assessment is constructed as a combination of the results of ecotoxicity tests and several fate-related physical-chemical and biological properties. This methodology is scientifically sound and in agreement with the present state of the art of ecological risk assessment procedures. Nevertheless, before going in a detailed evaluation of the capacity of this strategy to detect hazards related to endocrine disruption it is necessary to clearly identify its aims and the concerns considered under this system.

Ecotoxicological evaluations try to assess the effects of chemical substances at the ecological level. In scientific literature (e.g., Bro-Rasmussen et al., 1994), this level is commonly defined as "effects on the structure and function of the ecosystems" while due to the difficulties arising from the definition of the term ecosystem at the regulatory level (e.g., Gonzalez, 1996) the normative goal for ecological/environmental protection is frequently modified and other terms, such as "the protection of living organisms, environmental elements and their interactions", are used. In any case, from a scientific point of view both definitions clearly represent the aim to protect higher ecological levels of organisation than individuals-populations, such as communities and their interactions with the abiotic components of each environmental compartment.

However, it is not always easy to combine this theoretical "aspiration" with the "real" data source usually employed in ecotoxicological assessments, which in most cases is reduced to a limited number of laboratory single-species tests. This limitation is based on both technical and economic arguments. The technical arguments mostly focus on the difficulties for the interpretations of higher tier tests, e.g. aquatic mesocosms or terrestrial model ecosystems, while the economic arguments do not require further explanation.

The relevance of laboratory single-species (eco-)toxicity tests has been discussed elsewhere (e.g., Crossland, 1992). It can be considered as a pragmatic approach which is, nevertheless, widely employed as a cost/effective alternative (i.e., SETAC, 1994). From a methodological point of view is quite clear that these "ecotoxicity" tests are not "ecological" at all. In principle, we can assume that the test conditions of single-species (eco-)toxicity tests do not provide more "ecological information" than any (non-eco-)toxicity test on rats, mice, dogs, etc., which use a non-parenteral (i.e., oral or inhalation) route. A fish in a glass aquarium or an algae in an artificial reconstituted medium are not more, not less, ecologically relevant than a mouse in a box eating contaminated food.

In spite of these drawbacks, appropriate interpretation of these ecotoxicity tests results can lead to conclusions that are ecologically relevant. There are a number of factors that make this possible:

- the organisms used have been selected trying to represent key taxonomic groups of relevant environmental compartments, for example:
  - fish, invertebrates and algae for the aquatic environment,
  - plants, soil invertebrates and soil microorganismms for the soil compartment of the terrestrial environment.
  - vertebrates, pollinators and foliar dwelling invertebrates for the above soil compartment of the terrestrial environment.
  - top predators for biomagnification assessments.
- the toxicity endpoints are specifically selected to be considered as "ecologically relevant", i.e., reproduction, growth rate inhibition, etc. depending on the organisms and their ecological role.

- the interpretation of the laboratory information considers the "ecological goal" using either deterministic or probabilistic approaches to extrapolate the laboratory data to ecosystem effects. These interpretations require a specific discussion.

At the present state of the art of ecological risk assessment, deterministic or probabilistic approaches (based on laboratory single-species tests) can be justified not only as an economically feasible alternative, but can also be justified from a scientific point of view when a set of basic conditions, such as those included below, are fulfilled:

- these methods are part of a tiered approach, and constitute the first step of the process (lower tier), to determine if higher tier assessment are required. Their results are over-ruled when information at a higher tier level becomes available.
- the uncertainty of these assessments is quite high, and therefore requires the application of an appropriate level of precaution.
- the transparency of the process must be guaranteed.
- the decision schemes must be oriented to the reduction of type I-errors (minimising the risk for false negatives even by assuming a higher risk for false positives). A validation criteria for this condition is that higher tier values must show a clear tendency to reduce or at least to maintain the ecotoxicological thresholds estimated from the single-species toxicity data. The agreement between the recommended protocol and this validation criteria has been observed for several substances in the EU industrial and pesticide programmes.

Taken these and other considerations into account, the use of this approach is widely extended at the international level (i.e, OECD, 1989).

The deterministic approach is nowadays the most commonly used alternative in Europe. The ECETOC revision (ECETOC, 1993) compared methods developed in Switzerland, Germany, The Netherlands, the EC-JRC, UK, AIS and the OECD which were the basis for the development of the EC Technical Guidance Document (TGD). The ecotoxicological threshold is obtained by applying a factor (usually named as safety factor, uncertainty factor or application factor) to the lowest "relevant" toxicity value. This application factor depends on the significance and uncertainty of the available information. It should cover the extrapolation:

- from acute to chronic toxicity when acute toxicity data are considered,
- from laboratory to field conditions (unless laboratory conditions should be specifically designed to maximise the bioavailability),
- from the chronic effects observed for the most sensitive tested species to the long-term effects predicted for species-species interactions,
- from multi-species effects to the protection of the structure and function of ecosystems.

Typical examples of this approach are the derivation of :

- the EU Water Quality Objectives (Bro-Rasmussen et al., 1994).
- the Predicted No Effect Concentrations in the TGD for risk assessment of industrial chemicals (EC, 1996c).
- the OSPAR Ecotoxicological Assessment Criteria (OSPAR, 1994)
- the proposed Environmental Quality Standards in the Water Framework Directive (which follow the TGD estimation for PNECs)

Summaries of the application factors employed by the different methods can be found in ECETOC, 1993; OSPAR, 1994; Tarazona, 1998.

The probabilistic approach use the available information to produce a probabilistic distribution for the species sensitivity for each chemical. Obviously, the uncertainty in the estimation will depend on the amount and quality of the information employed to create the distribution. The ecotoxicological threshold is then calculated as the concentration which is safe for a predermined percentage (e.g., 95%) of the species (or other levels of taxonomic organisation). A classical example for this procedure is the Dutch Maximum Tolerable Concentration procedure (i.e, Van Straalen and Denneman, 1989).

Combination of deterministic and probabilistic approaches are possible in different ways, i.e. by applying a safety factor to the concentration which protect a certain percentage of the species (i.e., for the USEPA Water Quality Criteria, USEPA, 1995).

Nowadays, the tendency is to considered probabilistic approaches as a higher tier step in the tiered scheme. Several proposals for the incorporation of probabilistic methods in the EU Ecological Risk Assessment programmes for industrial chemicals and for pesticides have been presented.

It must be pointed out that in any case this approach tries to protect individuals or even species. If there is a concern for the effects on single species or even for the protection of individuals within a species (e.g. relevant for certain endangered species) then, as pointed out in section 4.7, the ecotoxicological approach is not valid and environmental (e.g. wildlife) toxicological methods are required. The outcome of this approach can be either more or less severe than the ecotoxicological method (e.g. requiring either higher or lower threshold values to obtain acceptable margins of safety), but in any case it can be assumed that individuals are protected by ecotoxicological thresholds.

*The role of single-species ecotoxicity tests in the assessment of endocrine disrupters.*
In theory, the basic concepts discussed above are general and not related to the mode of action of the chemical. Therefore any mechanism is expected to be covered in the extrapolation from laboratory data to the ecotoxicological threshold. This conceptual approach can be justified unless:

- due to technical problems the toxicity test/endpoints can be regarded as unable to detect the effects related to this mechanisms
- the uncertainty factors/probabilistic cut-off do not minimise type I-errors in the extrapolation from single species data to ecotoxicological thresholds (i.e. at the acute/chronic or chronic/multi-species ratio).

For some endocrine disruption mechanisms these “unless” clauses represent a potential problem, which cannot at present be quantified due to the lack of available information. As an example of technical problems, chronic studies on Daphnids (female populations with partenogenetic reproduction) and algae may not detect effects of oestrogenic pollutants, and the same can said for some (not all) chronic tests on fish such as the fish growth inhibition tests. However, the same chronic Daphnia test should be, in theory, a perfect candidate for the detection of androgenic pollutants.

Regarding the second clause, the most common extrapolation factor from acute to chronic effects (acute/chronic ratio) is 10, while not enough information is available to determine the ratio between mortality due to endocrine disruption and long-term ecologically significant effects due to endocrine disruption mechanisms.

In conclusion, although the conceptual approach is appropriate, and laboratory single species toxicity tests can be considered as either screening, lower tier or cost/effective alternatives for the prediction of ecological effects and independent, not related, to the specific mechanisms of action,

due to the particularities of the acute and chronic toxicity tests and endpoints currently selected, the hazard of some endocrine disrupters could not be identified by some widely used Ecotoxicity tests batteries.

Obviously the problem only appears when endocrine disruption is the only or the most sensitive and ecologically relevant mechanisms of toxicity and the effects cannot be directly or indirectly detected by the selected test endpoints in the scheduled time. Further information is required to estimate the magnitude of this problem.

The development of tests specifically designed to measure endocrine disruption, is not suitable for ecological effects because: a) the consequences must be evaluated at the population level, and b) even the smaller taxonomic groups (mammals, reptiles, amphibians) have thousands of different species with physiological and ecological differences which highly affect both the sensitivity and the ecological relevance of the effects observed at the individual level.

Particularly for endocrine disruption, our understanding on hormonal physiology is mostly limited to vertebrates, with some additional examples for some specific taxonomic groups. But even for vertebrates, it is difficult to predict the population consequences of individual endocrine alterations such us increases of vitellogenin levels or reductions in thyroid activities, until these alterations impair reproduction, growth or survival potentials. Therefore, the detection of these impairments must be the endpoints in ecotoxicity tests. Therefore, the ecotoxicological consequences of endocrine disrupters must be assessed by general, non specific endpoints but assuring that the employed tests and result interpretations fully cover the potential consequences of the hormonal alterations.

A potential solution should be to incorporate a procedure to identify the relevance of endocrine alterations. e.g. using the data produced on mammals. This hazard identification will be useful to select those tests which can produce the adequate response, e.g. to define which chronic toxicity tests on fish is required. This information should also be very useful for the assessment of secondary poisoning, this part is described as provisional in the current EU protocol for ecological risk assessment and although the potential for bioaccumulation is obviously included, additional efforts should be allocated for a better understanding of the risk related to the bioaccumulation potential of persistent chemicals and particularly for an appropriate characterisation of the potential for biomagnification through the food chains. A combination of potential for biomagnification and effects on reproduction and/or immunological capacity increase in an exponential way the risk for top predators, as can be clearly observed from the examples provided in the chapter on wildlife.

Finally, methods for an adequate characterisation of the potential risks of endangered wildlife requiring protection at the individual (for example Iberian lynx or Iberian imperial eagle) or population levels should be developed. These methods, to be developed as specific scenarios for the local risk assessment, should be applied on certain areas, in addition to the general ecological risk assessment, when endangered species are expected to be at risk.

# 6. Recent developments with respect to test guidelines and EDCs

## 6.1 Introduction

For regulatory purposes toxicological tests have to be carried out according to internationally accepted test guidelines. However, in several recent workshops and publications, it has been discussed whether the current test guidelines are suitable for identifying endocrine disrupters:

- SETAC-Europe/OECD/EC Expert Workshop on Endocrine Modulators and Wildlife: Assessment and Testing (EMWAT), Veldhoven, Netherlands, 10-13 April 1997;
- the OECD is at the moment finalising the Detailed Review Paper (DRP): "Appraisal of Test Methods for Sex-Hormone Disrupting Chemicals". The document has been prepared by the United Kingdom as a proposed basis on which to assess the suitability and availability of existing test methods used both by OECD member states and the research community. This document was circulated to the OECD National Test Guidelines Co-ordinators in April 1997 and has been recently revised to take account of comments received. A final document has yet to be published;
- a draft report from February 1998 of the activities of the Endocrine Disrupter Screening Testing and Advisory Committee (EDSTAC) of the US-EPA;
- a publication in Environmental Toxicology and Chemistry by Ankley et al. (1998) entitled "Overview of a workshop on screening methods for detecting potential (anti-) oestrogenic/androgenic chemicals in wildlife".

A summary of all tests available at the moment - including the recommendations for their enhancement with respect to endocrine disrupters - discussed in EMWAT, DRP and EDSTAC was presented by the OECD in a background paper of the first meeting of the OECD Endocrine Disrupter Testing and Assessment Working Group (EDTA), March 10-11, 1998. Test guidelines for invertebrates (OECD 202), fish (OECD 203, 204, 210 and 212 and the draft 28-days juvenile growth) and birds (OECD 205 and 206) and several tests with mammals like the OECD 407 and 416 are discussed therein. It is stated that none of the current OECD ecotoxicology test guidelines are specifically designed to detect endocrine disrupters. The same can be argued for the EU test guidelines as only regulatory test guidelines are available for acute tests with fish and daphnids, with non-specific endpoints, including growth and mortality.

The need for revision of existing OECD test guidelines and the development of new test guidelines specifically to address the potential adverse effects arising as a result of endocrine disruption, has been proposed by several organisations, such as EDSTAC, and in workshops on endocrine disrupters, such as EMWAT. Rather than discuss all existing test guidelines in the present document, it is decided to refer to the documents above.

## 6.2 Recent developments

Many initiatives have been taken in (inter)national fora to develop new test guidelines (*in vitro* screening as well as *in vivo* screening and comprehensive *in vivo* tests) and/or enhancement of existing test guidelines (additional parameters/endpoints in existing test guidelines). The most important initiatives are discussed below:

OECD Endocrine Disrupter Testing and Assessment (EDTA) Working Group

EDTA has been established jointly by the OECD Risk Assessment Advisory Body (RAAB) and the OECD National Co-ordinators of the Test Guidelines Programme (NC-TGP) in December 1997. The EDTA is the focal point for current OECD work on endocrine disrupters. In the draft report of the first EDTA meeting it is stated: "that specifically EDTA will provide (1) a forum to mutually inform national and regional activities, (2) develop appropriate OECD test guidelines and (3) where

possible harmonise risk characterisation and assessment approaches". EDTA recommends on the priority and future need for OECD work on endocrine disrupters by:

- "identifying and prioritising enhancements and modifications to existing OECD test guidelines to facilitate the detection of endocrine disrupting substances;
- developing a workplan for the development of top priority enhancements as indicators of endocrine disrupting effects, including an evaluation of their sensitivity and reliability;
- identifying and prioritising needs for new test guidelines and developing a workplan for future OECD work, including the validation of new test guidelines;
- developing a harmonised testing strategy for the screening and testing of endocrine disrupting chemicals taking into account the consequences of such a testing strategy on the development and validation of test guidelines, and on existing regulatory systems for new and existing substances".

The following non-mammalian tests were discussed in the first EDTA meeting:

*Mammals:* several tests - from in-vitro screening assays to higher tier tests like the two-generation reproductive toxicity study - were discussed. It was agreed that possible enhancements to detect chemicals capable of causing endocrine disruption should be considered.

*Birds*: It was noted by EDTA that the avian reproduction test (OECD 206) was currently in the process of being revised. It was agreed that possible enhancements to detect chemicals capable of causing endocrine disruption should be considered in the revision process.

*Fish tests*: A proposal to develop a fish full life-cycle test has been agreed by the NC-TGP. It was noted by EDTA that also the early life-stage test (OECD 210) has the potential to be able to identify an endocrine disrupter, provided the test is sufficiently enhanced. EDTA agreed that chronic testing for fish should be the subject of separate in depth discussions with appropriate experts. These discussions would aim to consider the various proposals for further fish tests - screening as well as confirmatory - and to recommend a suitable approach. As a first step an OECD Validation Management Group will be established, which will organize the development of fish test guidelines. EDTA agreed that a reference set of chemicals with a range of potencies for endocrine disruption should be identified for validation studies.

*Invertebrates*: It was concluded that no specific tests were available for endocrine disrupters in invertebrates. EDTA decided to await the outcome of a SETAC-Europe and North American/EC Expert Workshop entitled "Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment", to be held in December 1998. The provisional conclusions of this workshop highlight the importance of this taxonomic group, as invertebrates represent about 95% of the known animal species. The need for better understanding of invertebrate endocrinology and the large physiological diversity, which makes it scientifically unrealistic to select a "representative" species, were also stated. Regarding toxicity testing, the generation of data on several species is required, considering full life-cycle studies as the ideal bioassay. It was also pointed out that existing tests can be implemented for the detection of EDS effects by simply adding additional endpoints.

The CSTEE considers that the development of cost-effective multispecies laboratory tests, in which multiple reproduction strategies would be assessed simultaneously, will cover the identified needs and is realistic according to the current state of the art of invertebrate testing, and therefore requires priority.

*Amphibia*: EDTA concluded that there are no international accepted test guidelines available for amphibia. As EDSTAC has included a proposed amphibian development and reproduction test, it was agreed that the US would take the lead in developing a proposal for an amphibian test.

As a follow-up to the first EDTA meeting the "OECD workshop on the Validation of Endocrine Disrupters Screening and Testing Methods" was organised on August 10-11 of 1998 in the USA. One objective of the meeting was the selection of new test methods or enhancement of existing methods to be considered for validation. With respect to test methods the following was agreed:

- *mammals*: validation work has the highest priority for the 3-day uterotrophic, 5-7 day Hersberger assay and the OECD 407 repeated dose oral toxicity test in rodents (in general, the EU experts at the workshop were in favour of extension of the existing OECD 407, and not of the selection of new test methods).
- *wildlife*: expert workshops will be organised for fish testing (October 1998 in the UK) and mysid testing.

In this OECD Expert Consultation meeting on Testing in Fish (28-29 October 1998, London, UK) the following was concluded:

- work underway in various fora with respect to the development of methods for the detection of endocrine disrupters in fish is still at the level of "pre-validation". Test protocols for further guideline development could probably be submitted to a second expert consultation to be hold in September 1999.

The second EDTA meeting of November 12-13, 1998 focussed on tests with mammals. Protocols for OECD 416 (two-generation), enhanced OECD 407, uterotrophic and Hershberger assay were discussed. These will now be part of an OECD validation programme. A Validaton Management Group has in the meantime been established.

Endocrine Disrupter Screening Testing and Advisory Committee

The Safe Drinking Water Act (SDWA) Amendments of 1996 and the Food Quality Protection Act (FQPA) required US-EPA to "develop a screening program, using appropriate validated testing systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by naturally occurring oestrogen, or such other hormonal effect as the Administrator may designate". As a result of this EDSTAC was established. In October 1998 the final report was published. With respect to testing and test guidelines three phases - a tiered approach - are distinguished by EDSTAC:

*Pre-screening*: consists of use of (Q)SARs, information from the US-EPA Endocrine DisrupterDisruptor Priority Setting Database and High Throughput Pre-Screening (HTPS). In the HTPS 15,000 substances will be tested in an *in vitro* assay on oestrogen, androgen and thyroid receptor binding with and without metabolic activation.

*Tier 1 testing*: consists of the following tests: (1) *in vitro*: oestrogen receptor binding or reporter gene assay, androgen binding or reporter gene assay, steroidogenesis assay with minced testis; (2) *in vivo*: rodent 3-day uterotrophic assay, rodent 20 day pubertal female assay with thyroid, rodent 5-7 day Hersberger assay, frog metamorphosis assay and fish gonadal recrudescence assay. Possible substitutes are: (1) *in vitro*: placental aromatase assay; (2) *in vivo*: modified rodent 3-day uterotrophic assay, rodent 14-day intact adult male assay with thyroid and rodent 20-day thyroid/pubertal male assay.

*Tier 2 testing*: consists of the following tests: (1) two-generation mammalian reproductive toxicity study or a less comprehensive test (alternative mammalian reproductive test or one-generation test);

(2) avian reproduction; (3) fish life cycle; (4) mysid life cycle; and (5) amphibian development and reproductionrepoduction.

The non-mammalian tests recommended by EDSTAC are discussed below:

*fish gonadal recrudescence assay*: there are essential endocrine differences between fish and mammals: (1) fish differ in steroid profiles (11-ketotestosterone versus testosterone); (2) differences in oestrogen receptor; (3) steroid receptors in eggs for vitellogenin are unique for oviparous animals. In this assay fish of both sexes maintained under simulated winter conditions are exposed to an increasing photoperiod, temperature and test substance to determine effects on maturation from the regressed position (recrudescence). EDSTAC recommends the fathead minnow (*Pimephales promelas*) as test species.

*avian reproduction*: EDSTAC recommends the EPA Avian Reproduction Test Guideline - test species are the mallard duck and northern bobwhite quail - to be enhanced with some additional observations: steroid titrestiters, organ and gland weights, histochemistry and histopathology, and reproductive capability of the offspring.

*fish life cycle test*: EDSTAC recommends the fathead minnow (*Pimephales promelas*) or the sheepshead minnow (*Cyprinodon variegatus*) as test species, depending on whether the substances will lead to exposure of freshwater or estuarine/marine systems, respectively. Test organisms will be continuously exposed from fertilisation through development, maturation and reproduction, and early development of the offspring (test duration up to 300 days).

*mysid life cycle test*: endocrine disrupters can interfere with ecdysteroid activity, being an important steroid in arthropods. In this test the effects on development, molting, growth and sexual reproduction are studied.

*amphibian development and reproduction*: in this test the effects on amphibians exposed from the larval stadium through metamorphosis and reproduction is studied.

With respect to the validation status of the tests recommended EDSTAC distinguishes 5 categories:

Category I: tests which have been fully validated and standardised;
Category II: tests which have been in use for a sufficient period of time and have gained sufficient general acceptance. Standardisation should be accomplished.
Category III: tests which have been used sufficiently broad to be generally considered relevant or reliable. Further validation and standardisation is necessary.
Category IV: tests which may be relevant, but have not been used very often. Method development, validation and standardisation is necessary.
Category V: tests which have actually not been conducted yet. Research should be carried out to determine whether these tests can be developed, and determine which purpose they can have in the endocrine disruption screening and testing program.

The following non-mammalian tests were placed in these categories:

Category II: avian reproduction, fish life cycle and mysid life cycle;
Category IV: fish gonadal recrudescence;
Category V: avian androgenicity screening test, invertebrate screening tests, avian multi-generation test, amphibian development and reproduction test and reptilian test.

Endocrine Modulator Steering Group

In June 1996 the chemical industrial organisation in Europe CEFIC - the (Conseil Européen de l'Industrie Chimique) - established the Endocrine Modulator Steering Group (EMSG). After a public call in The Lancet for tenders, a research programme was announced in a press release on 14 May, 1998. The EMSG programme is part of a global programme of the chemical industry, in which the Chemical Manufacturers of America (CMA) and the Japanese Chemical Industry Association (JCIA) are the other key players. With respect to wildlife EMSG will focus on fish, while the CMA concentrates on birds and reptiles. At the EDTA meeting in March 1998 the EMSG presented three proposals on tests with fish:

- *in vivo* screening test: juvenile fathead minnows (*Pimephales promelas*) will be exposed for 21 days. Endpoints are induction of the egg yolk precursor vitellogenin and sex steroid levels.
- modified early life-stage (ELS) tests (enhanced OECD 210): randomly selected fathead minnow will be held without further exposure until maturity and subsequent egg-laying. In addition histological analyses of the gonads (presence of oocytes in testicular tissue and incorporation of yolk into oocytes) and biochemical analyses (vitellogenin, sex steroids and analysis of genetic sex) will be performed.
- partial life cycle test: sexually mature adult fathead minnow will be exposed for 28 days. Biological observations (daily counting of fertilised/unfertilised eggs) and histological analysis of the gonads (see above) will be carried out. On one occasion eggs will be collected. Eggs and hatched fry will then be exposed for 28 days post-hatch and then follow the same procedure as described above for the ELS test.

In the EDTA meeting it was discussed that also other fish species than the fathead minnow could be used for this type of toxicity testing.

# 7. Conclusions and recommendations

## 7.1 Human health effects

Exogenous substances or mixtures may cause adverse health effects in humans primary or secondary to changes in endocrine function, given that such exposures are large enough to damage maintenance of homeostasis, reproduction, development and/or behaviour. Although there are associations between endocrine disrupting chemicals, so far investigated, and human health disturbances, a causative role of these chemicals in diseases and abnormalities possibly related to an endocrine disturbance has not been verified. Assessed from relative potencies based on oestrogen receptor interactions, steady-state concentrations of endocrine disrupting chemicals in normal human serum samples are several orders of magnitude lower than those of endogenous hormone levels. However, a number of the compounds implicated do not exert their effects via the oestrogen receptor. At present the following conclusions can be made:

- a meta-analysis of 61 studies has reported a general decrease in sperm concentration and semen volume from 1938 to 1990. However, several reanalyses of the same data have indicated possible bias and confounding in the meta-analysis, and have reached different conclusions with respect to sperm quality, depending on the methodology used. Recent, well designed studies have shown that there are large regional differences in overall sperm quality and time trends, both within and between countries.
- a time-dependent increase in the prevalence of cryptorchidism has been reported in several investigations, but the studies are difficult to compare due to differences in design and examination techniques. There also appears to be an increase in the prevalence of hypospadias in several European countries, but the studies may suffer from lack of proper ascertainment. No causative role for endocrine disrupters for the increased prevalence in cryptorchidism or hypospadias has been determined.
- the incidence of testicular cancer has increased significantly during the last 30 years, with an increase of 2-4% in men under the age of 50 in northern European countries. There is a 5-fold difference in the age specific incidence of testicular cancer among 25-34 year olds in Denmark (high) vs. Finland (low). The underlying reason(s) for the increased incidence in testicular cancer has not been identified.
- there also has been recorded an increased incidence of prostate cancer in Europe during the last decades. Any causative role for endocrine disrupting chemicals in development of prostate cancer has not been established.
- there has been a steady increase in breast cancer incidence rates over the last decades in Europe. There is almost a 2-fold difference between countries with the highest (Switzerland) and lowest (Spain) incidence rates. The available data associating breast cancer development with exposure to organochlorines do not support a causal relationship.
- prevalence estimates for endometriosis give an average of 10 per cent; the etiology of this disease is unknown.
- there have been several reports on the declining proportion of male new-borns during the last decades; this decline in sex ratio remains unexplained.
- high accidental exposure to PCB of pregnant women have led to delays in physical and mental development of the offspring resembling hypothyroidism. There are indications that lower exposures to organochlorine compounds may affect neonatal neurological development, possibly by affecting thyroid hormone status. There is no convincing evidence linking exposures to endocrine disrupters and thyroid cancer development.
- the reported health effects which have been associated with endocrine disrupters should be further evaluated and the underlying causes identified. In this, special attention should be given to exceptional high chemical exposures and to the health consequences of phytoestrogens in human food.

## 7.2 Effects on wildlife

It is evident that many wildlife species may be exposed to biologically active concentrations of endocrine disrupting chemicals including a large number of persistent organochlorines, natural and synthetic steroid hormones, organotins, alkylphenols. There is strong evidence obtained from laboratory studies showing the potential of several environmental chemicals to cause endocrine disruption at environmentally realistic exposure levels. In wildlife populations, associations have been reported between reproductive and developmental effects and endocrine disrupting chemicals. Effects have been observed in a broad number of species representing many major taxa including mammals, birds, reptiles, fish and molluscs from Europe, North America and other continents. Most of these studies refer to aquatic food chain organisms, only few to terrestrial systems. The observed abnormalities in various wildlife species vary from subtle changes to overt and permanent structural and functional alterations, including disturbed sex differentiation with malformed (feminized or masculinized) sex organs, changed sexual behaviour, and altered immune function. Impaired reproduction and development causally linked to endocrine disrupting chemicals are well documented in a number of species and have caused local or regional population changes. These include:

- Marine snails. Imposex is probably the clearest case of endocrine disruption caused by an environmental chemical. This phenomenon occurs in many species of gastropods and correlates with harbours and shipping lanes. The proven causative agent is tributyltin, a biocide used in anti-fouling paints. The dogwhelk appears particularly sensitive and the development of imposex has resulted in decline or extinction of local populations worldwide, including coastal areas all over Europe and the open North Sea.
- Birds. DDE-induced egg-shell thinning is probably the best example of reproductive impairment that caused severe population declines in a number of raptor species in Europe and North America. A large number of predatory birds have suffered from reproductive and developmental toxicity caused by persistent organochlorines which biomagnify in the food chains. Developmental exposure to the DDT complex has been firmly linked to the induction of ovotestis in male Western gulls.
- Fish. There is convincing evidence that endocrine disrupting chemicals have adversely affected a variety of fish species. Only in the vicinity of certain sources (e.g. effluents of water treatment plants, wood- and paper processing industries, textile industry) and in the most contaminated areas is this exposure linked with reproductive organ effects which could have implications for fish populations. However, there are recent indications for a more widespread occurrence of oestrogenic effects in the United Kingdom, where significant oestrogenic exposure of fish is not restricted to freshwater systems, but also occurs in estuaries and coastal areas.
- Mammals. The best evidence comes from the field studies on Baltic grey and ringed seals, and from the semi-field studies on Wadden Sea harbour seals, where both reproduction and immune functions have been impaired by PCBs in the food chain. Reproduction effects resulted in population declines, whereas suppression of immune function have likely contributed to the mass mortalities due to morbillivirus infections
- Reptiles. Distorted sex organ development and function in alligators has been related to a major pesticide spill into a lake in Florida, U.S.A. The observed oestrogenic/anti-androgenic effects have been causally linked in experimental studies with alligator eggs to the DDT complex present in the formulation.

For most other reported effects in wildlife, however, the evidence for a causal link with endocrine disruption is weak or non-existing. This is mainly due to the complexity of contaminant mixtures, the lack of chemical exposure data, of data on the sensitivity of the species concerned, and of knowledge on mechanisms of action. Crucial in establishing causal evidence for chemical-induced

wildlife effects are semi-field or laboratory studies using the wildlife species of concern. Although most observed effects currently reported concern heavily polluted areas, there is a potential global problem. This is exemplified by the widespread occurrence of imposex in marine snails and the recent findings of high levels of persistent potential endocrine disrupting chemicals in several marine mammalian species inhabiting oceanic waters. The CSTEE recommends:.

- to assess the full environmental significance of endocrine disruption, including studies on effects in terrestrial systems and in amphibian and reptile populations in Europe.
- to conduct further field, semi-field and laboratory studies in order to establish cause-and-effect relationships. To infer such relationships is complex and difficult, since poor reproductive performance and subsequent population change is multi-factorially defined. Also many other environmental and stock-related factors such as habitat destruction and fisheries impact may influence population structure and size. In this respect it is important to establish baseline data in non-exposed reference populations.
- to encourage the establishment of co-ordinated biomonitoring programmes by international monitoring organizations (e.g. OSPAR, HELCOM and MEDPOL), the European Environment Agency and national environmental agencies to identify the hazards of endocrine disruption to aquatic and terrestrial wildlife. In doing so, consideration should be given to the strategies and guidelines for the design and conduct of such programmes provided by two international Expert Workshops EMWAT and EDIETA.
- to establish the long-term consequences at the population and community level of the observed effects in reproductive organ structure and function (such as vitellogenin induction, precocious female maturation, intersexuality, ovotestis formation, and altered sexual behaviour), thyroid status and immune system.

### 7.3 Ecological risk assessment

Ecological risk assessment is intended to evaluate risks on the structure and functioning of ecosystems. Endocrine alterations at the individual level can or cannot produce consequences on populations, communities and ecosystems. The strategy for ecotoxicity assessment must focus on relevant endpoints for the detection of population-community effects. Risk assessment protocols mostly follow tiered approaches. Any relevant endocrine disruption effect is expected to be identified when high tier level are used. However, this high tier levels are only employed when low tier evaluations detect potentially unaceptable risks. The analysis of current protocols for ecological risk assessment indicates a concern on the capability of low tier levels to detect the ecological risk of endocrine disrupters due to:

- methodological problems such as the suitability of the test species (i.e. partenogenic organisms that are not appropriate for the assessment of oestrogenic substances).
- epistemological uncertainties (i.e straightforward extrapolation from acute lethality to long-term effects).

Moreover, some wildlife species (in particular endangered species) require an additional level of protection, which in some cases much reach the protection of individuals. This level of protection must be considered as a complement, not an alternative, to the ecosystem protection. Therefore, the concerns on the capability of the current testing strategy and hazard and risk assessment protocols to detect and properly consider all relevant effects associated to endocrine disruption must be evaluated. The CSTEE considers that it is a priority:

- to establish the capability of laboratory long-term tests to detect ecologically relevant effects related to endocrine disruption.
- to determine which margins of safety and uncertainty factors must be considered, in the case of endocrine disrupters, for the extrapolation of ecotoxicological thresholds from acute and chronic toxicity studies.

- to consider when species-focused assessments may be required and to develop decision making schemes and risks assessment procedures for critical wildlife species which require a complementary level of protection, such as endangered species.

### 7.4 Toxicological test guidelines and testing strategies

Based on the present review of ecotoxicology and toxicology regulatory test guidelines, the CSTEE makes the following conclusions/recommendations:

- present regulatory toxicology test guidelines, in particular the guidelines for ecotoxicity testing, cannot detect all endocrine disrupting effects. Therefore, current test guidelines have to be enhanced or new test guidelines developed. For this purpose proper knowledge on the endocrine system of the species concerned is needed.
- the current enhancement by the OECD of the existing 407 repeated oral toxicity test in rodents and the existing OECD 416 reproduction toxicity test has full support, and is preferred over the development of new test methods. Both tests are included in the base-set for new and existing chemicals in the EU. Promising methods are the uterotrophic assay and the Hersberger assay, but they need further development and validation.
- for fish, it is recommended to enhance the early life-stage test and to further development the partial life-cycle test. Besides these screening assays, the confirmatory full life-cycle test needs development. In these tests histopathological and biochemical parameters should be included. The implications of toxicological enhanced vitellogenin synthesis on fish reproduction should be clarified.
- for birds, the present one-generation test guideline should be enhanced; no screening and two-generation test guidelines are available.
- for invertebrates, the existing tests, e.g. mysids, should be implemented by the inclusion of appropriate endpoints which cover full life-cycle effects related to endocrine disruption. Testing strategies should include several invertebrate species. Combined-species laboratory tests or integrated multispecies exposure systems with single species constitute alternatives.
- *in vitro* assays may be useful in setting priorities for further testing and for supplying information for understanding the mode of action. However, utilising *in vitro* assays for predicting *in vivo* endocrine disrupter effects may generate false-negative as well as false-positive results. Thus, major emphasis should be put on *in vivo* assays.
- international co-operation (EU, OECD, EMSG) in the development and validation of test protocols is essential to avoid duplication.
- for the active substances of new plant protection products testing with an updated version of OECD protocol 407 and 416 should by asked for. Active substances already on the market but not tested according to these testing system should be priorities and tested accordingly.
- for new chemicals produced in less than 1 tonne/year it should be considered whether there is a need to additional toxicity testing for reproductive toxicity (currently only if >1 t/year) or fertility (currently only if >10 t/year). In that case, new criteria for decision should be developed that are also based on structure-activity relationships.
- the testing strategy for ecotoxicological assessment should be implemented with a hazard identification step for endocrine disruption if potential for endocrine disruption activity is indicated using structure-activity relationships or mammalian toxicity tests.
- for new and existing chemicals the requirements for toxicological and ecotoxicological data on reproductive effects should be harmonised and the technical guidance document updated with focus on testing strategy for endocrine disruption.

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