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Endocrinically Active Chemicals in the Environment

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At the invitation of the German Federal Environmental Agency 80 scientists from universities, research institutes, agencies of the Federal and Laender governments, from industries and environmental organizations participated in the expert round "Hazards by Endocrine Chemicals in the Environment“ on 9. and 10. March 1995. The aim of the expert round was the discussion about the occurrence and impact of substances that have an endocrinic effect, and on potential risks that may arise for human and the environment. Papers covering several aspects of this topic were presented.

Papers on the hormonal effects of environmental chemicals on humans focused on the following topics:

- Trends in the number of sperms in males; indications of increased cryptorchism,
- concentration of natural and synthetic estrogens in various environmental compartments including sewage and drinking water,
- effects of DES (diethylstilbestrol), in particular, on descendants of women who were exposed during pregnancy,
- effects of phytoestrogens (substances from plants having an estrogenic effect) and their concentrations in foodstuffs,
- effects of dioxins (especially TCDD) at intrauterine exposure, in particular, on fertility and sexual behaviour,
- effects of dioxins (especially TCDD) and polychlorinated biphenyls (PCB) on sperms and trophoblasts (embryos prior to implantation), endometrial functions and concentrations of hormones,
- estrogens and tumorigenity (esp. cancer of the testicles and the breast).

Papers dealing with action in other organisms focused on the following topics:

- occurrence and effects of endocrine substances in fish,
- problems related to assessing the impact of chemicals in the environment on sex differentiation of fish,
- sex distribution trends for a North Sea fish over the past 14 years,

- € effect of 3,4-dichloroaniline on the androgen metabolism in fish,
- € effect of organotin compounds on snail populations,
- € methods for determining the estrogenic potential of chemicals in the environment.

The following conclusions were adopted after intense discussion:

The possibility of an environmental influence on the hormonal control in organisms is scientifically indisputable.

At present it is impossible to establish cause-effects relationships to explain detectable defective developments.

Potential effects on humans and on nature are so tremendous that there is a need for immediate clarification.

Progress relating to scientific studies that aim to clarify the role of estrogens for the development of organisms is most advanced.

The following four classes of compounds should be considered when studying endocrinic effects on humans and animals:

1. natural estrogens,
2. synthetic estrogens,
3. phyto- and mycoestrogens (substances in plants and fungi that have an estrogenic effect),
4. chemicals in the environment.

Effects in humans

A number of phenomena has been observed in epidemiological studies and register inquiries of humans. The cause of these phenomena should be further explained. They include:

reduced fertility
increased occurrence of breast cancer

Furthermore there are indications for an increase of testicular cancer. The increased occurrence of cryptorchism and hypospadias that is being discussed requires further verification.

Apart from alterations in reproductive capacity, animal studies indicate that sexual behaviour may also be affected.

Effects in animals

Sex is not a priori determined in a great number of fishes. Sex differentiation is influenced and controlled, on the one hand, indirectly by abiotic and biotic factors and, on the other hand, directly by synthetic hormones and most probably also by chemicals in the environment. In addition, synthetic hormones in aquatic cultures are used intentionally for economic purposes, e.g. to preserve "monosex cultures". Field sampling and field observation in Germany has not yet provided any hints that give rise to general conclusions about the impact of harmful substances on the development of sex in fish; long-term investigations have shown, however, that there is a slow, but significant decline in the proportion of females among dab (*Limanda limanda*) populations e.g. in the North Sea.

Experiments with 3,4-dichloroaniline have shown that industrial chemicals have an effecton the hormonal system of fish.

Synthesis of vitellogenin (yolk proteins) in male fish seems to be a sensitive parameter that is suitable for determining estrogenic and anti-estrogenic effects in fish. This test showed activity of low concentrations of alkylphenols. It was weaker than that of natural estrogens but occurred at environmentally relevant concentrations. Increased vitellogenin synthesis has been observed, along with unspecific toxicity, in fish in British sewage treatment plants; occurrence of alkylphenols is discussed as a possible cause of this. Additive effects of the substances in wastewater cannot be excluded. The relevance of the vitellogenin test system for population biology should be checked.

As to molluscs, clear drops in population density have been observed in many snail species due to imposex (formation of male sex characters in females that can result in sterility). These effects were found in prosobranchs in waters contaminated with tributyl tin. Exposure analyses corroborated the hypothesis that the risk for these populations can mainly be attributed to the occurrence of tributyl tin and perhaps other organic tin compounds. They increase the testosteron level in the animals.

Little is known about the half-life and action of substances that have an endocrinic effect in the terrestrial system. It may be assumed that soils, to a considerable extent, serve as a sink for hormonally acting substances.

It is important to note that potential hazard for human and animals may result not only from exposure to estrogenic and anti-estrogenic substances but also from exposure to other hormonally active chemicals, as androgenic and anti-androgenic substances.

Recommendations

The following procedures were suggested to determine factors for quantifying the risk that arises from hormonally acting substances:

1. and 2. Natural and synthetic estrogens

Collection of reliable data on exposure to natural and synthetic estrogens, their active metabolites and the relevant routes of entry.

Available data indicate that natural and synthetic estrogens are detectable, for example, in the groundwater, in drinking water and in flowing waters. A comprehensive assessment seems to be necessary taking into account the ranges of activity of the various compounds and metabolites.

3. Phytoestrogens

Evaluation of human exposure to endocrinically acting plant constituents taking into account veterinary knowledge. Groups with specific nutritional habits (vegetarians) seem to be particularly exposed.

4. Chemicals in the environment

The problem of chemicals in the environment is the most complex, both in terms of the number of substances and in terms of possible effects. It is safe knowledge that a great number of chemicals in the environment have a potential of interfering with hormonal control circuits. More research is urgently required to answer the question whether, and to what extent, this poses a risk for man and the environment. Participants speak out for a nationally and internationally coordinated and interdisciplinary approach to studying in depth the toxic and ecotoxic effects of a small number of endocrinically acting model substances to describe possible endpoints and to develop screening methods. Existing routine procedures, especially procedures for detecting alterations in reproduction and

development, should be reviewed and, where necessary, modified to cover the above endpoints.

Above all, research strategies should be developed to determine bioavailability and possible combinatory effects in target tissues. There are indications of additive effects with environmental estrogens. Additive effects of the various chemicals should therefore be included in the assessment.

Individual substances and mixtures of substances such as wastewaters, liquid manure, etc. are to be examined for their endocrinic activity using biological test procedures. Existing test methods should be combined where suitable to ensure that impacts on the hormonal system are detected in an optimum way and as comprehensively as possible.

Main Points of Discussion

Intensive discussions were held after a paper had been presented, and again at the end of each day. This chapter summarizes the main points of discussion.

Effects in humans were discussed on the first day

The problem whether findings from animal experiments can be transferred to humans was raised in connection with effects on human fertility. It is a specific problem to assess reductions in the number of sperms in humans as they carry a considerably smaller surplus of sperms than the animals used in the experiments (double surplus in man against a 10-fold surplus in rats). It was made clear by the participants that there is no causal relation between oligospermia and fertility but that motility of the sperms is another important factor. In addition, the sociocultural background should be taken into account when a couple has fertility problems, for example, that couples tend to plan children at a higher age today.

Increased occurrence of breast and testicular tumors has been observed in various industrial nations. This was brought about, apart from exogenic factors, by improved diagnostics and more frequent preventive medical examinations. But it was considered likely that exogenic factors (food, environment) are predominant.

Participants agreed that only the estrogenic activity of many substances is known, whereas androgenic effects are not examined. Studies of the effect that a substance has on a hormonal system are impeded by the fact that compounds such as dioxin may cause opposite effects in different organs.
Available exposure data were felt to be insufficient. It was pointed out that conjugated estrogens are normally overlooked when assessing exposure with regard to estrogens that were released in the environment by human or animal excrements. Detection methods for conjugates need further improvement. Collection of individual component data, however, is a major condition for comprehensive evaluation. In the light of today's knowledge, a reinterpretation of the findings of the 1979 water, soil and air study (Rathner and Sonneborn, Forum Städte-Hygiene 30 (1979), pp. 45-49) is justified. Wastewater analyses and a projection for the city of Kiel e.g. showed concentrations of ethinyl estradiol that were above the threshold value for the induction of vitellogenin synthesis in male fish.

As far as drinking water and surface waters are concerned, the question about what maximum levels are permissible was left open. It became clear that differing strategies can be followed

depending on the object to be protected and the goal of protection. Natural and synthetic estrogens can be detected in drinking water down to the lower nanogram range. Direct effects on humans in this range have not yet become known. It is therefore a matter of political assessment to decide whether, similar to pesticides in drinking water, limit values should be set.

The discussion about the exposure of humans and animals via the food chain revealed that phytohormonal effects as studied in veterinary medicine have hardly been dealt with in human medicine. Soy and maize products, in particular, contain relatively high concentrations of phytoestrogens. Babies with a milk allergy could therefore be exposed to a greater extent because such products are used as a substitute for milk products. However, it is currently impossible to evaluate the importance of this finding. The recent workshop of the "Toxicology Forum" in the U.S.A. gave particular attention to the question of exposure to phytoestrogens via foodstuffs (Fawell and Wilkinson, J. Water SRT-Aqua 43 (5), pp. 219-221, 1994).
If humans take in endocrinic agents with their food, the question on potential effects of natural estrogens occurring in milk arises.
The subsequent discussion dealt with uptake kinetics of synthetic substances as compared with natural steroids. Natural steroids occur as conjugates in a bound form in the human body and are therefore unable to pass through the placenta or the blood-brain barrier. Synthetic substances, on the contrary, are not bound to proteins and may bring about pre-natal exposure. It is being discussed that this could have an impact on "imprinting", the irreversible processes during the embryonic development of the central nervous system, as alterations in sexual behaviour have been observed after pre- or perinatal exposure.
The endocrinically active substances under discussion have shown an efficiency in in-vitro tests that is 10^{-3} to 10^{-6} times lower than that of estradiol. It was pointed out that the respective substances have not yet been systematically studied in a test system so that any comparison of their relative activity based on individual values from different sources has its uncertainty components.

Effects of endocrinically active substances on aquatic organisms were the primary topic of discussions on the second day of the expert round

Papers presented showed that field sampling and field observation allow only limited statements on the development of sex in fish as the result can be severely influenced by factors such as the fishing technique or the size distribution of male and female fish as a function of their age.

The discussion frequently returned to the question of appropriate test systems and parameters to detect endocrinic activity. The in-vitro test methods described in the literature (receptor assay, vitellogenin synthesis in fish hepatocytes, proliferation of human tumor cells) do not reflect the endocrinic activity of a substance but only its direct estrogenic effect. Chemicals in the environment such as 3,4-DCA or TBT would not be recognized hazardous if only these test systems were used.

Endocrinic activity of substances cannot always be predicted from their structure. As the example of TCDD shows, predictions of membrane permeability or bioaccumulation can only be made to a limited extent.

Another point of discussion was the relevance of suitable test systems for humans. It should be checked in each individual case whether findings can be transferred to other species.

Some of the participants pointed out that the proliferation test using the MCF-7 human tumor cell is the most suitable method out of the presented in-vitro test procedures to determine estrogenic efficiency. But there was agreement that this test is not yet sufficiently stable to be used as a standard test.

As to in-vivo test systems, the suitability of imposex induction in whelks was discussed. The objection was that up to now, with the exception of ethanol, only tin organic compounds was examined, and that therefore suitability of this parameter was not yet established. In addition, the steroid metabolism of prosobranchs is insufficiently known. Proliferation of the head kidney in sticklebacks was presented as an easily identifiable indicator of a disturbed androgen metabolism. Experience is still limited to just a few compounds, however.
It was also mentioned that another method could be exposure of fish eggs and observation of their development. Suitability of a fish life cycle test was discussed controversially. Some participants rejected the test as being too lengthy and expensive. Others, however, considered it a suitable system to validate new test procedures.

It was discussed whether the induction of vitellogenin synthesis in male fish by chemicals in the environment and wastewater is a suitable test parameter. But it remained open whether this parameter has any relevance in terms of population biology, for example, whether it is a reliable indicator of a damaged population. This test showed activity of alkylphenols at low concentrations. The drain channels of sewage treatment plants also showed positive reactions that were explained in the literature as being due to alkylphenols. This observation becomes

important as nonylphenol is present in the environment at concentrations just short of their effects concentration (NOEC for daphnia reproduction 10 g/l).

The outcome of the discussion was that the observed phenomena can only in exceptional cases be associated with individual substances (TBT, high degree of contamination by PCB, TCDD or DDT/DDE). Additional measures related to individual substances therefore cannot be defined at present (considering PCB and DDT are prohibited and TCDD emissions were dramatically reduced in Germany). General prevention can only be achieved by reducing discharges of persistent and accumulating substances into the environment.

Hormonally Active Substances in the Environment: A Preface

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The question whether chemicals in the environment can influence the hormonal system of humans and animals was first discussed in our agency in the 1970s. The centre of interest at that time were hormones in human and animal excrements. Estimates revealed that natural hormones and hormones from pharmaceuticals are introduced into the environment. The final analysis concluded that there was no need for action.

But when scientists found that not only natural hormones and pharmaceuticals with a similar structure could influence the hormonal system but also pesticides, industrial chemicals and heavy metals, and that these substances can cause disorder of the endocrine system, the topic had to be taken up again.

Various effects in humans and animals are attributed to hormonally active substances, for example, reduced fertility, deformations of the genitals, development of cancer and defects of embryonal development at the stage of sex differentiation.

There are numerous hints and suspicions, especially in the United States, but a clear cause and-effect relationship could rarely be proved.

This workshop is to give an overview over the current state of research, point out knowledge gaps, and thus contribute to arriving at a research strategy; last but not least, it is to help to show ways in which preventive protection of the environment and the consumers can meet the need for protection of the public and of nature - despite the fact that scientifically accurate answers will not be achievable for all questions.

This workshop will try to provide answers to the following questions:

1. **What is the extent of environmental contamination caused by endocrinicall y activesubstances?**

   Many observations were made in highly contaminated areas, above all, in the United States

   What is the extent of contamination in Germany and in Europe?

2. What is the share of the following classes of substances in this contamination:

- **natural sexual hormones of human and animal origin**
- **synthetic sexual hormones**
- **endocrinically active chemicals (agonists and antagonists)?**

Is there any proof of joint action of these substances?

Potential cumulative effects of the various substances that are active in the environment should be taken into consideration.

3. **Does exposure of humans influence fertility?**

   Recently, there has been a controversial discussion of retrospective studies on male fertility. What is the value of such studies?

4. **Do endocrinically active chemicals increase the risk of cancer, in particular, of breast cancer in women? Are there indications of an increased risk in descendants?**

   Delayed effects of pre- and perinatal exposure are of particular interest here.

5. **Do endocrinically active substances influence fertility or sex differentiation in such a way that there will be a negative impact on species and ecosystems?**

   Where in Europe are indications of an impact on ecosystems? Do endocrinically active substances contribute to endangering species?

6. **Should hormonal activity increasingly be taken into account when evaluating chemicals?**

   Are there sufficient test and evaluation strategies (Toxic Substances Control Act, Plant Protection Law) to detect delayed effects in descendant generations?

7. Judging from today's knowledge, should further measures be taken to ensure preventive environmental and health protection?

A number of regulations has entered into force in recent years, all of which aimed at reducing the discharge of substances that are suspected of having an endocrinic effect (e.g. prohibition of PCP, tighter limits for dioxin emissions, prohibition of PCB). Are more such measures required?

Estrogenic Chemicals in the Environment: A new Problem?

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Knowledge that xenobiotics act in a similar way as hormones, and the resulting ability of influencing endocrinic regulations, is not new. It has been known since the 1960s, for example, that apart from plant constituents (phytoestrogens) described much earlier, synthetic compounds such as methoxychlorine, DDT, and polychlorinated biphenyls may develop estrogenic activities in laboratory animals (see table 1). In 1979 and 1985, symposia on the subject "Estrogens in the Environment" were held in the United States discussing the ecotoxicological and toxicological relevance of estrogenic substances in the environment (6,7).

Tab. 1: Description of estrogenic effects of xenobiotics

1936	bisphenols	Dodds & Lawson (1)
1954	genisteine	Bradbury & White (2)
1961	methoxychlorine	Tullner (3)
1968	DDT	Bitman et al. (4)
1970	PCBs	Bitman & Cecil (5)

The recently increased interest in the problem of estrogens in the environment can be explained by three items: 1) recent findings on sex differentiation and reproductive biology in game populations including e.g. fish, reptiles and birds (8), 2) results of experiments using fish as bioindicators in draining sections of British sewage treatment plants (9), and 3) recent epidemiological studies on the development and function of human genitals, especially of males.

Studies related to item 3) point to a reduction in sperm production and an increase in incidents of testicular cancer, cryptorchism, and possibly hypospadia over the past 30 to 50 years.

1. Sperm production

A Danish working group conducted a statistical analysis on the results of 61 investigations on sperm density in ejaculate of men, published between 1938 and 1990. Amongother things they concluded from the results that the mean sperm density decreased from 113 x 10^6/ml in 1938 to

66 x 10^6 in 1990 (10). Although this study partly gives rise to justified criticism with respect to the adopted methods, there are arguments that a decrease in sperm production has taken place during the last decades. This is supported by the results of other independent examinations with men from Sweden, Denmark and France (11-13).

Figure 1 summarizes the results of the different studies. Out of the publications of Carlsen et al (10) only those with 100 and more test persons are included (open squares). With the exception of the data from Bostofte et al. (median values) arithmetic mean values are shown.

Figure 1: Sperm densities between 1938 - 1992, investigations with n > 100.

2. Testicular cancer

Several investigations conducted in different countries give evidence for an increase in testicular cancer incidence. For example, the age-normalized incidence has more than doubled in Denmark between 1943-1982, and it has quadrupled in the age group of 15-24 years (14).

3. Cryptorchism

According to investigations performed in England the incidence of cryptorchism of 3-month-infants has nearly doubled between the 50s and 80s (15).

4. Hypospadias

An increase in incidence of hypospadias, postulated by several authors, at present cannot be proved by appropriate investigations.

Referring to experiences with the synthetic estrogen diethylstilbestrol (DES), administered to a large number of pregnant women in the 40s to 80s, a hypothesis was proposed recently saying that the above-mentioned disturbances of the male reproductive system could have a common cause, that is, increased prenatal exposure to estrogens.

Principally, an exposure to both, endogene as well as exogene estrogens is possible.
Table 2 summarizes factors possibly contributing to a raised estrogen exposure in utero.

Tab. 2: Factors possibly contributing to an enhanced estrogen exposure in utero

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A) Endogene estrogens

1. nutrition low in roughage € enterohepatic circulation

2. increase in body fat
   - estrogen synthesis in the fatty tissue
   - decreased SHBG-synthesis in the liver

B) Exogene estrogens

1. synthetic estrogens
    - medication of pregnant women with DES
    - DES-addition to fattening forage for animals
    - oral contraceptives in the drinking water?

2. phytoestrogens
    - for instance soybean

3. environmental pollutants with estrogen effect

The different factors cannot explicitely be addressed to in this article. Regarding exogene estrogens, apart from synthetic estrogens and phytoestrogens estrogenic environmental chemicals have to be considered. It is still unclear whether relevant concentrations of excreted metabolites of oral contraceptives (ethinylestradiol) may reach sewage waters resulting in contaminations of surface waters and ground water. Likewise unclear are possible consequences of an enhanced uptake of phytoestrogens due to special nutrition habits. Moreover, not enough knowledge is available at present to allow an assessment of the importance of endocrinic environmental pollutants; respective assessments are complicated by the fact that estrogenic environmental pollutants present only a part of the total endocrinic active substances occurring in the environment.

Estrogenic activity has been determined for a number of substances of different chemical classes (examples in fig. 2). Even though the experimentally determined estrogenic potential of all these substances is several orders of magnitude below that of 17 €-estradiol, it cannot be concluded from the experiments that they are biologically inactive. This especially applies for persistent estrogenic substances which accumulate in bodies of humans and animals and are possibly being biotransformed to metabolites of even higher activity. The situation is still complicated by the fact that some ubiquitous environmental chemicals possess an antiestrogenic potential.

Considering the importance of the observed statistical trends concerning sperm production, testicular cancer, and cryptorchism there seems to be urgent need to prove the hypothesis of an enhanced prenatal estrogen exposure. This would however require considerable research efforts.

Fig. 2

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Excretion of Natural and Synthetic Estrogens and Their Metabolites: Occurence and Behaviour in Water

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Natural estrogens are mainly formed in the ovary, during pregnancy in the placenta and moreover in smaller quantities in the adrenal glands and testicles. In general they control the development of the secondary female sex characteristics and together with the gestagens they regulate almost all of the reproductive processes in women.

17-ß-estradiol, the main secretion product, is formed from testosterone via 19-OH-testosterone The natural estrogens have an (very stable) aromatic ring A and a phenolic hydroxyl group in position 3. They are transported in the blood bound to plasma globulins or albumins, metabolized in the liver and excreted in the urine or via the bile and intestines. From the intestines they can be reabsorbed again.

A woman's daily estrogen secretion is 25-100 µg depending on the menstrual cycle; this quantity can rise to 30 mg/day at the end of pregnancy. But estrogens are produced not only in humans, all the animals are estrogen-producers, too. A pregnant mare, for example, produces 100 mg/day.

Figure 1 : Metabolism of natural estrogens :

Figure 1 shows the metabolism of 17-ß-estradiol. The hormone is first oxidized to estrone, which plays a central role in the further process. One metabolic pathway results in ring D metabolites, from estrone via 16-€-OH-estrone to estriol, which can be - under certain circumstances - the main metabolite. A second pathway leads via ring A substitution to the so called catecholestrogens; 2-OH-estrone is shown here as an example, which can partly be further metabolized to 2-OMe-estrone. Further metabolites can be formed by ring B substitution. Examples for this are : 6-ß-OH-estrone, 6-oxo-estrone and 6-€-OH-estrone (1). All of these metabolites will be conjugated in the liver to estrogen-sulfate-esters or estrogen-glucuronides. Moreover there are double and mixed conjugates as well. Both types of molecules are acids, they are much more polar, but above all they are much more water-soluble, than the unconjugated estrogens. They will mainly be excreted in the urine. The absolute amount and relative composition of the metabolites may vary depending on the menstrual cycle and health (compare in Table 1: in column 1: 44% ; in column 2: 52% ; in column 3: 41% are ring A metabolites. On the contrary in column 4: 91% are ring D metabolites) (3,4).

Table 1: Excretion of estrogens in the urine (µg/day):

	pre-menopausal	post-menopausal	males	during pregnancy
2-OH-Estrone	11,5	8,5	4,9	670
Estrone	8,0	4,0	3,9	600
Estriol	4,8	1,0	1,5	19980
2-OH-Estradiol-17ß	3,6	0,7	0,6	82
Estradiol-17ß	3,0	2,3	1,5	259
4-OH-Estrone	2,5	2,0	0,9	-
2-OMe-Estrone	2,5	1,3	1,2	52
16-€-OH-Estrone	2,2	0,6	1,2	3650
16-€-OH-Estrone	0,9	0,6	0,7	-
16-OXO-Estradiol-17ß	1,5	0,7	0,6	2088
16-Epi-Estriol	1,5	0,5	0,8	586
€	**€ 42**	**€ 22**	**€ 18**	**€ 28000**

Fotsis et al. (1980) and (1987)

The natural estrogens are orally inactive or only at higher dosage active, since they are quickly metabolized. More active and above all more stable compounds were developed by the introduction of an ethinyl-group in position 17-€ of the estradiol molecule. Ethinyl estradiol and its 3-Me-ether mestranol are the most frequently used estrogen components in contraceptives. Via the introduction of the ethinyl-group the ring D becomes extremely stabilized against

oxidation. Thus, estrone - which plays a central role in the metabolism of the natural hormons- cannot be formed here. The consequence of this increased stability is, that ethinyl estradiol is excreted up to 80% unchanged in conjugated form (2).

Once the natural or synthetic estrogens are excreted in form of their conjugated metabolites, they will be found in wastewater. During the biological sewage treatment, wastewater meets a mixed population of microorganisms (so called activated sludge), the task of which is to reduce the amount of organic compounds under aerobic conditions. Laboratory experiments with optimized cultures and added nutrients (5) have shown that it takes several weeks, before no estrogens are detected in the system anymore (Table 2). The most stable molecule in this system was ethinyl estradiol.

Table 2: Loss of natural and synthetic estrogens in activated sludge (%):

	weeks			
	1	2	3	4
Estriol	81	89	97	100
Estradiol	90	96	100	
Estrone	94	98	100	
Ethinylöstradiol	73	82	90	95

Tabak & Bunch (1970)

Another study (6) found that ethinyl estradiol and mestranol remained in activated sludge fully unchanged over 5 days (Table 3). Table 3 shows that the synthetic gestagens - chemically much less stable than the natural estrogens - are much faster decomposed than ethinyl estradiol. Presumably, the stability of the natural hormones might be somewhere in between of them. Since by the latter experiment activated sludge and wastewater taken from a sewage treatment plant, without added nutrients were used, its biological circumstances were closer to reality than that of the previous one.

Table 3: Stability of synthetic estrogens and gestagens in an activated sludge model (%):

	16	24	36	48	72	96	120 (h)
Norethisterone acetate	23	-	-	-	-	-	-
Chlormadinone acetate		40	-	-	-	-	-
Norgestrel		30		8	-	-	-
Lynesrenol		58		42	10	-	-
Megestrol acetat				39	19	-	-

Medroxy progesteron acetate		30	22	8	-
Mestranol	100	100	100	100	100
Ethinyl estradiol	100	100	100	100	100

Norpoth et al. (1973)

There are no studies available that could reveal the fate of the conjugatedestrogen metabolites in wastewater. From a chemical point of view these conjugates are fairly stable. At the beginning of the estrogen metabolite research hot acid hydrolysis was used to release the free estrogens from their conjugates, a procedure, during which a considerable part of the free estrogens became immediately decomposed. However, the estrogen conjugates can carefully be cleaved by enzymatic hydrolysis as well. The enzymes, that can mildly cleave the conjugates are quite often isolated from microorganisms, which might be present in wastewater as well. But it is not determined yet, how active these enzymes under these circumstances are.

Table 4: Estrogen concentrations in water samples (ng/l) :

wastewater, agriculture	135 - 350
wastewater, Tel Aviv	54 - 135
purified water	6.5 - 50
after sandfiltration (3 months)	2.7

Shore et al. (1993)

Table 4 shows estrogen concentrations measured in wastewater. This study (7) was made in Israel and shows the influence of farm animals as well. The keeping of large stocks of animals can lead to locally increased estrogen concentrations. It can also be seen that through the sewage treatment plant a reduction of the estrogen concentration could be achieved. Unfortunately the sand filtration value does not tell us what to the adsorbed estrogens happened. These values were measured by RIA using a 17-ß-estradiol antibody, that had 25% cross-reaction with estrone. Since it is known, that these two molecules can reach only about 30% of the total amount of metabolites (excreted by humans) and during pregnancy only 3 - 4%, the real burdens of the estrogen contaminations might substantial be higher than the values shown here.

The investigation of 56 drinking water samples from springs and wells for estradiol and ethinyl estradiol in southern Germany yielded in 1977 the results summarized in Tables 5 and 6 (8).

These values were measured by RIA. Since estradiol is only a minor part of the estrogen metabolites, it is to be assumed that the total estrogen values have been much higher. The ethinyl estradiol concentrations were evidently higher than that of estradiol. This illustrates the higher stability of ethinyl estradiol. Ethinyl estradiol concentrations of =1ng/l were measured in several of these drinking water samples, a concentration that showed definite endocrine effects in fish in laboratory experiments (9).

Table 5: Estradiol concentrations in drinking water (ng/l):

	number	mean value	range
springs	9	0.30	0 - 0.82
springs und wells	8	0.18	0 - 0.52
wells bis 20 m	7	0.42	0 - 0.94
wells (20-60 m)	12	0.16	0 - 0.33
wells (60-100 m)	13	0.17	0 - 0.72
wells unter 100 m	7	0.12	0 - 0.80

Rurainski et al. (1977)

Table 6: Ethinyl estradiol concentrations in drinking water (ng/l):

	number	mean value	range
springs	9	2.89	0.34 - 14.20
springs and wells	8	1.52	0 - 3.95
wells to a depth of 20 m	7	3.18	0.70 - 9.20
wells (20-60 m)	12	2.94	0 - 22.50
wells (60-100 m)	13	0.96	0 - 4.50
wells below 100 m	7	0.85	0 - 1.90

Rurainski et al. (1977)

Exact informations on the behaviour and occurence of the natural and synthetic estrogens are lacking. Estrogen conjugates, too, can be biologically active. Conjugated estrogen metabolites are, for example, isolated from pregnant mares' urine and used in human estrogen therapy even up to date.

Summary

1. Natural and synthetic estrogens (as conjugates) enter the wastewater in considerable concentrations.
2. Particularly synthetic estrogens are stable enough to withstand the sewage treatment process.
3. Measurements made up to now show that estrogens may occur in surface waters and even in drinking water in detectable concentrations.
4. In general it is surprising, how little is known about the occurence and behaviour of estrogens in the environment.

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Exposure to, and Activity of, Estrogens: Knowledge and Experience Gained with the Synthetic Estrogen Diethylstilbestrol (DES)

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Diethylstilbestrol (DES) has been administered to millions of pregnant women in the United States and in Europe from 1947 to the 1980s. It was wrongly assumed that it could help to preserve pregnancy and to prevent miscarriages. The orally active synthetic estrogen DES and similar substances were prescribed at massive doses: 5 mg per day, in most cases increased by another 5 mg per day every other week, i.e. 125 mg/day in the eighth month of pregnancy, were quite common [1]. For comparison, see the quantities of the estrogen component contained in oral contraceptives (e.g. ethinyl estradiol: 30 to 100 µg/day).

Herbst, Ulfelder and Poskanzer found clear cell vaginal adenocarcinomas in 7 young women at an age from 14 to 21 years for the first time in 1971 and attributed this finding to DES intake of their mothers. This cause-and-effect relationship was clearly confirmed by extensive studies carried out later. They also revealed dose-action relationships and particularly critical stages of prenatal exposure. In addition, laboratory animal models were developed that showed similar pathologic changes like those found in humans after in-utero exposure to DES.

Many consequences of a DES exposure are known today [2]. The relative risk that so-called DES daughters develop an adenocarcinoma of the vagina and the cervix at a young age is estimated to be 1 in 1,000. But the percentage of women who developed vaginal adenosis or in whom were found other, non-cancerogenic alterations of the genital tract is much higher. At present, it cannot be stated with certainty whether this group is likely to show late effects such as an increased tumour risk after the menopause. 30% of "DES sons" showed various types of abnormalities of the genital tract, e.g. cryptorchism and epididymal cysts.

An increased breast cancer incidence was reported for the group of "DES mothers", which was noticeable only after a considerable latency period (≥ 20 years) [3].

Many studies on the mechanism of action of DES have been carried out, including studies of the question whether similar adverse effects should be expected, for example, for steroid estrogens. As these studies cannot be presented in detail here, let me just refer to the findings and hypotheses that could be of importance for a risk assessment of other (endocrinocally active) substances.

(1) DES has virtually the same in-vitro affinity for estrogen receptors (ER) as estradiol (E_2), a physiological ligand, and has a comparable estrogenic efficiency in vivo. Somewhat elliptically, one can conclude that DES act as a hormone; (all) its activity is mediated by the E-receptor. The decisive factor for adverse effects, e.g. the transplacental cancerogenic effect, is the influence of high doses at the wrong time. It results in a differentiation defect that is expressed later.

(2) DES (like other cancerogenic chemicals) can be metabolized into reactive intermediates that bind to cellular macromolecules. Its metabolites can cause oxidative stress and the respective DNA modifications. Though DES has not proved to be mutagenic in various short-term assays, it was found to be genotoxic [4]. It induces micronuclei and aneuploidy, and causes neoplastic transformation of (ER-negative) cells. It can be concluded that other activities not controlled by E-receptors play an important part as regards adverse DES effects. It is unknown as yet whether these become relevant at relatively high concentrations only.

(3) In the Syrian golden hamster, a laboratory animal in which kidney tumours are induced by DES [5]. DES treatment can affect the metabolism of foreign substances, in particular, some cytochrome P-450 activities. Whether the metabolism of endogenous steroid hormones is also modulated under these conditions has not been investigated. Still, indole-3-carbinol and polycyclic hydrocarbons can induce P-450 isoenzymes that catalyze the 2-hydroxylation of E_2 or estrone, thereby reducing their estrogenicity. These examples are to

illustrate another way, that is, indirect mechanisms that contribute to modulating the activity of endogenous hormones.

The three mechanisms described above are not mutually exclusive. This means, for example, that it may be a short cut to assess hormone-mimetic substances based on receptor affinity only. Substances that accumulate in the body such as kepones are more efficient in vivo estrogens than would be assumed on the basis of their weak receptor binding. Other substances such as o,p´-DDT or DMBA are transformed in the organism into metabolites with stronger estrogenicity [6].

Apart from synthetic compounds, natural substances are known which possess estrogenic activity, for example, mycotoxins and phytoestrogens. Exposure of humans to the latter at levels that reach endocrine efficacy can be expected. Alterations in the length of the menstrual cycle and maximum LH/FSH levels were reported for women who ate 60 g of soy protein (with 45 mg of daidzein) [7]. Whether these effects are rather due to an antagonistic mode of action has not yet been clarified.

Recent publications [8, 9] have dealt with various aspects of risk assessment of hormone mimetic xenobiotics and natural substances. Although opinions about the toxicological relevance of such substances, especially for humans, are quite diverse researchers agree that there is a great need for further studies in this field.

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Possible Consequences of Pre- or Early Postnatal Exposure to Substances with Estrogenic or Androgenic Properties

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1. Summary

Adverse effects on reproductive components and functions are extremely complex. They may be induced by many factors, and are certainly not confined to chemicals. In this survey, special emphasis is placed on aspects of the male reproductive system and fertility, which by no means implies that substance-induced effects on the female reproductive system are of lesser importance. There are many ways to interfere with components and functions of the male reproductive system. Such causative factors include: diseases, psychological factors, hormonal variations and substance-induced effects. Effects of xenobiotics may be induced directly or indirectly. Directly means by a direct interaction of the substance with components or reactions of the reproductive system. Such possibilities are assessed in routine studies (segment I or multigeneration approach). Indirect interference could result from alterations of hormonal regulations. They will only be detected with the routine methods presently used if they clearly interfere with fertility. This can only be expected at high dose levels. Both types of effects, direct and indirect ones, have almost exclusively been revealed from animal studies.

Based on results from animal or *in vitro* studies, interference with hormonally-regulated reproductive functions due to effects caused by high doses of environmental substances has been described for almost 25 years now. Such substance-induced alterations of the reproductive system may be triggered at any stage of development, ranging from the fetal stage to the adult organism. An impressive amount of data on this topic is available [1].

More recently, the possibility of disturbances induced during the pre-/perinatal period attracted special attention. While many adverse effects on the adult male organism are reversible, pre- or perinatally-induced effects on the male (and also female) reproductive system may be irreversible. Although this area has not been systematically explored, some results from rat experiments have been published describing irreversible effects on the morphology and function

of sex organs, sex hormonal regulation, and sexual behavior after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) *in utero* or during lactation. These results constitute the only available extensive data base on substance-induced effects, and therefore these data will be discussed in this paper in some detail. It will be shown that even this data base is quite fragmentary, no clear-cut dose-response relationship has been established, and many questions still remain unanswered. While suggestive of an interference with hormonal regulations, it is largely unknown whether this effect induced by TCDD is a direct one on the development of certain sexual functions, or the result of an antihormone action of TCDD on defined sexual organs and functions during development. Furthermore, if it is hormonal, it is not known whether it is due to an estrogenic or an antiandrogenic action, and it can only be speculated on the possible mechanism of action.
Although it might be possible to offer some speculations, it has to be stressed that the relevance of all these data from animal studies for the situation possible existing in humans is completely unknown, and the data available until now are unsuited and insufficient for a human risk assessment. There is no information available on no-observed-adverse-effect-levels (NOAEL) in the rat, the significance of the data from rodents for the situation possibly existing in primates remains obscure, and it is impossible to draw general conclusions, e.g. as to the potency of other congeners or related substances.

If this aspect of reproductive toxicology is considered to be relevant for a risk assessment for men, considerably more basic as well as applied research in this field is necessary before far-reaching conclusions may even be attempted. In order to perform the extensive studies necessary, substantial funding is essential. Such studies are not facile (there are only a few institutions with enough expertise in this field), they are expensive, time-consuming and labor-intensive to carry out, and the results cannot be interpreted at all if the studies are insufficiently designed. An increased awareness on the part of regulatory agencies in Germany, coupled with the necessary financial commitment could finally permit initiation of such long delayed, but much needed studies.

2. Introduction

For several decades possible substance-induced carcinogenic actionsdominated the interest in toxicological studies and data compilations. More recently, interest and concern shifted to conceivable substance-induced effects on reproduction and development. Since this is the

largest and most complex field of toxicology and numerous aspects are still undisclosed, concentration of toxicological concern on these functions is quite understandable.

There are a few recent hints pointing to possible changes in components of male reproductive performances. These include conflicting data on the possibility of decreased average sperm counts in humans over the last decades [e.g. 2]. Despite the fact that numerous explanations for such deviations are feasible, and although there is at present insufficient evidence of such a trend, it has become fashionable to speculate on a possible connection of such deviations with environmental exposures, specifically to substances with estrogenic properties. If such overall deviations and changes in physiological and pathological variables in the populations of several countries should turn out to be true and might possibly even indicate a beginning trend towards clearly adverse effects, closer analyses of all possible causes certainly are justified and needed.

There are numerous factors that might modify or alter reproduction and fertility in humans [3,4,5,6]. These include:

- Diseases (e.g. certain viral diseases),
- Stress and psychological factors,
- Nutrition and physical factors (e.g. temperature),
- Substance-induced effects.

While there are many indications pointing to the first three mentioned factors, information concerning substance-induced adverse effects on reproductive components, sexual behavior, and fertility in humans are scarce and not well proven (with the exception of effects induced by DBCP, by cytostatic agents, and by hormones [1]).

There is no doubt that a variety of chemicals have the potential, as deduced from animal studies, of interfering with components and functions of the reproductive system. In fact, it is part of the routine preclinical investigations required by regulatory agencies to test for possible substance induced effects on male and female fertility. Among many possibilities to affect sexual reactions and behaviors, i.e. predominantly direct effects on the gonads, also disturbances by indirect means, e.g. by interference with hormonal regulations, are feasible. By far the best information on such possibilities comes from studies on synthetic hormonal substances, like those with estrogenic, androgenic or with antiestrogenic or antiandrogenic properties. Vast information on experimental as well as clinical data is available on the effects of these substances, and all we have learned in this regard stems from studies on such synthetic derivatives of sex hormones, primarily developed for therapeutic purposes. In contrast, information on substances with "ecohormonal" properties is quite fragmentary, and no reliable data suitable for any kind of risk assessment for humans are presently available. So far, data were obtained with systems of various complexities, pharmacokinetic data are almost completely lacking, especially with respect to human exposure, thereby not allowing an evaluation of the special situation possibly existing in man.

Among such effects are possible substance-related actions on the **prenatal development** of sexual organs and functions, especially if the substances (mostly at high exposures) exhibit some effect on hormonal (estrogen or androgen) receptors or possibly even modify the hormonal response at the specific receptor. Such hormone-dependent pre- or early postnatal processes might be especially relevant and interesting, and they might exhibit a special vulnerability to defined types of substances. However, it is fair to state that our present

knowledge on such substance-related effects is quite rudimentary, and there are only few rather extensively investigated experimental examples for such a potential. The reasons, why such data are either difficult or impossible to interpret are manifold, ranging from methodological shortcomings, inadequate documentation of the results obtained so far, to the inability to extrapolate such data to the situation possibly existing in primates and thereby in humans due to inadequate data on species differences and similarities.

It appears worth mentioning, although probably not relevant to this discussion, that there are also publications, especially from Denmark [7,8], reporting an increase in certain testicular tumors, and this trend has been linked to environmental causes as well.
We shall confine the discussion to the possibility of substance-induced permanent effects induced either during fetal development or during the early postnatal period. The principles of such effects on the development of the male reproductive system, as they are known today, and the few data available on substance-induced effects will be briefly discussed. This information is put into perspective with certain preliminary findings in connection with the human male reproductive system. Of course, it is impossible to cover all important aspects in a short review.

We would like to put forth some questions and try to answer them, we will comment on the information available and also on our present lack of knowledge in this field.

3. Is there sufficient evidence of a decrease in human sperm quality in the general population over the last five decades?

Answer: *No. Although a considerable number of publications exists on this topic, there are not sufficient data available to allow the conclusion that human sperm quality is declining in the general population. The reports on this matter are quite conflicting. However, based on the data available, such a possibility can also not be excluded (especially if it occurred in the nineteen-forties to nineteen-sixties).*

Comment: The main source on which to base such a claim, is a meta-analysis of 61 reports on human semen quality published between 1938 and 1991 [2]. The papers reviewed by these authors are of widely differing quality, on groups between 7 and 4,435 men from various countries. All of the data do not represent information on the general population, but they are from highly selected groups of men. While for most of the earlier studies detailed and sufficient

information is lacking and the evaluation was confined to mean values (a poor choice since a non-linear distribution is well-established), rather well-documented data are available from recent studies [e.g. 9]. There are many shortcomings inherent to such attempts of an multi-paper evaluation, and there is general agreement that the data available are insufficient to arrive at the conclusion of a temporal decrease in human semen quality in the general population.

The crucial question is whether the data sets available may suggest a **trend** in the reduction of motile sperm in human semen. The term "trend" would give a hint on a possible continuous decline which might also be assumed to continue in the future. This is certainly the main concern, since it must be taken as an alarm signal for possible changes to clearly adverse effects in the future.

In several comments it has been criticized that it is not justified to apply a linear regression to the data base, as was done by Carlsen et al. [2]. There is really no indication that a decline (if it is real and not caused by confounding factors in the highly selective groups studied) has occurred linearly. On the contrary, the data compiled [2] rather point to a decline during the years 1940 to about the mid-sixties, and after this no clear-cut change could be deduced from the data cloud of the mean values spread over an extremely wide range. Thus, the data are really not sufficient enough to speculate on a change within the last 15-20 years, neither an increase nor a decrease.

Such a conclusion seems to be contradicted by the data of Auger et al [9], reporting a decline in the concentration and motility of sperm during the last 20 years. This report contains by far the best-documented data available, depicting all individual data. However, also in this data set it seems obvious that a linear regression is not compatible with the data. Many of the intrinsic difficulties with this type of data can best be recognized in these well-documented results, and they will be discussed in some detail.

The wide range of the values for sperm density of the men with proven fertility (only those were included) is striking (original data in figure 1A). Furthermore, the year by year variability becomes obvious in this data set. It is also strange that during the first four years of the data collection no values < 8 Mio sperm/ml were found. However, these first four years contribute most to the slope of the linear regression. If the first four years are omitted (figure 1B) and the more appropriate medians are considered, at first glance there seems to be no decline present (a closer analysis with the original data not available to us has to be performed).

There is another possibility to evaluate the data (figure 1C): by assuming a middle reference range and considering the values above this range. Interestingly, the portion of men with rather high sperm concentrations (i.e. > about 300 Mio/ml) does not seem to change within the study period. This would either indicate that the study population was extremely divergent and could hardly be compared, or that there was no obvious change. There were a number of men in the later years of the study (but not between 1973 and 1976) who had fathered at least one child, but had at the examination < 5 Mio sperm/ml. This was apparently a random event, since it occurred at several years over the entire study period. There also seems to be no change during the period studied with respect to the scattered data cloud at the lower part of the graph, but these data are less representative since only fertile men were selected. Overall, there seems to be no linear change in sperm concentration over the years studied: without being able to analyze the individual data, it still appears that e.g. there is no difference between the years 1977 and 1979, and 1987 and 1989. Instead, there is a considerable yearly fluctuation, probably due to the wide range covered by the data.

When comparing the data of individual years, it is difficult to postulate a continuous change Thus, also the data of Auger et al. [9] do not provide clear-cut evidence of a change within the last 16 years. A claim of a decline in sperm concentration and especially of a continuous **trend** within the last 15 years does not seem to be supported by the data available today.

Table 1: Variability of the sperm concentration in Himalayan rabbits (Thomae, spf).

Semen quality was measured twice a week (Tuesdays and Fridays) for prolonged periods. Each column gives the individual data for one rabbit at various days. These are selected data out of 60 rabbits studied in a similar way. Indicated is the *inter-* and *intra*individual variability (in Mio sperm/ml).
[Unpublished data: Chahoud/Neubert 1982].

Semen analysis #	Rabbit # 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8
1	420	255	225	1290	540	765	975	1800
2	240	870	210	1410	1710	495	630	225
3	765	990	570	675	705	360	105	570
4	90	765	300	150	765	885	1995	1950
5	1515	255	120	1020		780	4425	405
6	285	525	555	345		60	1020	915
7	585					675	900	1005
8	1440					825		1305
Median	*503*	*645*	*263*	*848*	*735*	*720*	*975*	*960*
Mean	*668*	*610*	*330*	*815*	*930*	*606*	*1436*	*1022*

There are several problems concerning the methodology used in these studies. A crucial one is, whether it is at all possible to obtain useful data from studies in which single semen specimens

are collected from the volunteers. It is well-established from animal data that day by day there is a tremendous variability in semen samples collected from the same individual. Table 1 shows some of the rabbit data we obtained in our laboratory. In an experimental animal study one would not dare considering single control data (0-time data) as providing any information on the "*sperm count of this individual*". It is quite clear that before initiating an experiment many samples must be collected from each of the individuals, and only evaluations on a statistical basis are meaningful. When a larger number of samples are collected over a long enough period of time, median values become rather representative for this individual, and only then are statistical evaluations, e.g. with a subsequent treatment period, possible. It can also be seen that there is no normal distribution of the data, and the mean values are sometimes close to the median, but certainly not always.

Also some human data have been published on this aspect. The semen quality of seven healthy sperm donors (aged between 19 and 46 years at the end of the collection period, mean ± SD: 32 ± 9 years) was measured over a period of 72 to 324 weeks. Between 61 and 205 specimens were collected from each volunteer [10]. All had fathered children. Besides a large *inter*individual variability, there was a huge *intra*individual range in the sperm concentration and ejaculate volume for each of the normal men (table 2). When the profile of the semen quality of the person donating 205 specimens over a period of 324 weeks is evaluated (figure 1 of that paper), the tremendous fluctuation is quite obvious. Since this day to day variability also applies to the semen volume and the percentage of motile sperm, the most important variable for assessing the reproductive capacity, i.e. *number of motile sperm per ejaculate*, is bound to vary extremely in individual sperm samples on a day by day basis.

Table 2: Variability of semen quality in normal men.

Semen quality was measured in 7 healthy semen donors in 61 to 205 samples pro volunteer over a period of 72 to 324 weeks.
Sperm concentration is given in million sperm/ml, the ejaculate volume in ml.
M ± SD [Data from: Mallidis et al. 1991].

Characteristics	Volunteer						
	# 1	# 2	# 3	# 4	# 5	# 6	# 7

Number of specimens	61	62	83	80	77	105	205
Sperm concentration	51	78	116	156	128	86	124
(range)	*17-176*	*9-239*	*28-335*	*61-376*	*24-318*	*23-188*	*16-317*
Ejaculate volume	2.3	3.9	3.4	2.5	2.0	3.1	2.7
(range)	*0.5-4.8*	*1.5-5.6*	*1.5-6.2*	*1.0-4.0*	*0.4-3.5*	*0.7-5.5*	*0.5-5.0*
Percentage motility	74%	71%	72%	70%	73%	71%	69%
(range)	*45-94*	*32-88*	*50-91*	*52-86*	*54-90*	*50-89*	*37-94*

Thus, serious doubts are justified as to whether data on single specimens per individual are of any scientific value at all, and whether any type of serious conclusion can be based on such inadequate data. It is recognized that the data basis available is small and the data far from being ideal. However, the argument of methodologically and biologically insufficient data cannot possibly be invalidated by any statistic enterprises. No statistics can achieve a conversion from insufficient to acceptable data. This also applies to any attempt of a "meta-analysis". An approach like that is only meaningful when comparable data of good quality are assessed together, the main argument being that each of the (acceptable) studies contains too small a number of individuals. These, and several other prerequisites are not met in the reports on semen quality published over the last decades. *In any additional, and preferentially prospective, future studies these serious objections must be taken into account*.
Summarizing, there have been a number of publications reporting a decline in sperm number per ml seminal fluid in man [9,11]. In other reports such a trend could not be found [12,13] indicating the difficulty of far-reaching conclusions and of judging the validity of such data.

Many more data are required to arrive at any justified and reliable conclusion. Since the matter might be medically relevant, and the public has already been alarmed, further studies should be initiated. This will certainly not be an easy task. It could be suggested to analyze more of

available data sets if information on more than one semen specimen per individual is available Furthermore, prospective studies on unselected groups of men should soon be started However, this would not solve the problem of a possible decline that occurred in the nineteen-forties to nineteen-sixties, even if we assume a steady state condition now.

4. What about the general possibility that a possible decline in human sperm concentration is caused via toxic influences?

Answer: *There is no doubt whatsoever that toxic, substance-induced influences have an impact on sperm production and semen quality. There are numerous examples from animal studies but very few (and rather speculative) examples in humans (exception: DBCP).*

Comment: Since there is not sufficient evidence that there is a continuous trend with respect to a reduction in sperm concentration in the general population, considering a possible causal relationship would mean taking the second step before the first one.

However, the problem of substances being in our environment which possibly exhibit toxic properties on male fertility or sperm quality is significant enough in itself. Thus, this problem should be uncoupled from the hypothesis or speculations discussed before.

We have to recall what the purpose of toxicological studies, experimental as well as studies in humans, is: detecting and defining possible hazards, assessing the possible relevance for humans, and providing the basis for primary or secondary prevention.

For establishing and using experimental data there are four quite different approaches which are of equal significance but require different methodologies and different strategies:

1. Obtaining clues to a possible toxic potential of a substance as a hint to specifically consider these endpoints in the human trials. In this case, finally the human data are the only important ones.
2. To provide data for primary prevention. Toxic effects (such as carcinogenicity, reproductive toxicity, mutagenicity) are revealed which cannot easily or only after long lag periods be verified or excluded in humans. The experimental data will be used for

decision making with respect to possible human hazards. If it is decided to largely or completely avoid exposure of humans, we will never know what the real toxic potential of this substance in humans is. However, this will be of little interest.

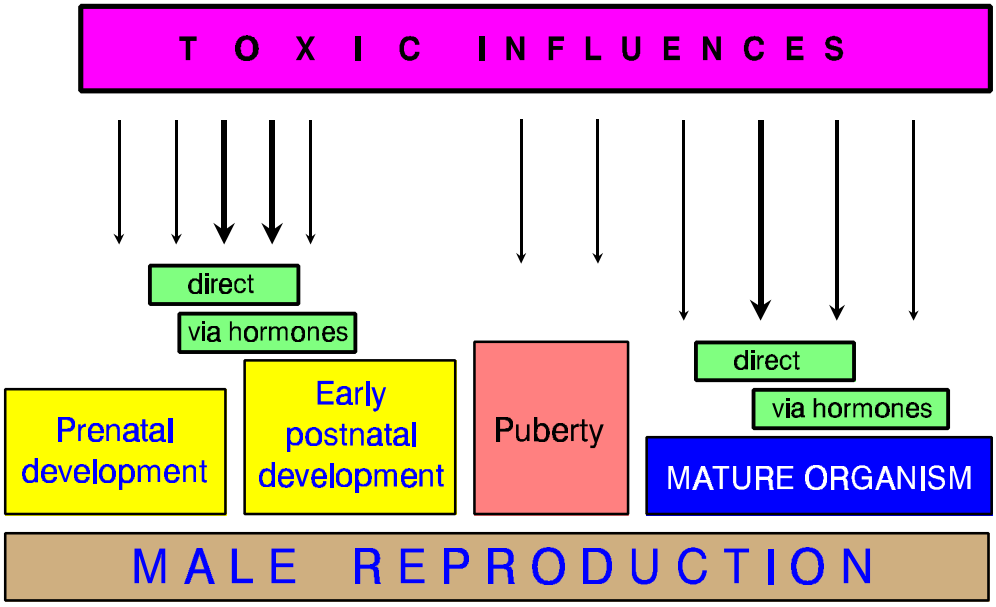

Figure 2: Possible points of attack on components and functions of male reproduction . Adverse effects may be triggered starting at prenatal development, up to the adult organism . Adverse effects may be induced directly on male sex organs, or indirectly via an alteration of the hormonal regulation.

3. Assessing the possibilities of an already existing toxic potential (the extent of which is unknown, may also be negligible). This concerns e.g. the body burdens with substances of high persistence (dioxins, DDE, etc.). It is expected from experimental research to answer the following questions:

   (a) What may be an underlying mechanism, and which endpoints must be considered?

   (b) What are the susceptible periods for such effects in general and what are they specifically for the substance in question?

(c) Which types of substances exhibit which types of toxic actions?
(d) What is the dose-response, what is the possible no-observed-effect-level (NOEL or NOAEL)?
(e) Which tissue concentrations occur at a given toxic effect or at a NOEL?
(f) What is the body burden of humans? Is there a chance that toxic levels may be reached?

In subsequent studies in humans this information must be taken into account for purposes of generating a hypothesis. A population with an extended body burden must be recruited and possible effects evaluated against a group with considerably lower body burdens. Individual kinetic data are essential for such an evaluation.

4. It is also possible to obtain first clues to possible effects from observations in humans. Collaboration of colleagues from clinical fields with scientists working experimentally will facilitate reaching the goal of establishing possible cause-effect relationships and of making a meaningful risk assessment.

With respect to the **possible points of attack** on variables **of male fertility**, substance-induced interference is possible with several developmental stages (figure 2):

- Certainly, on the adult organism.
- On developing organs and functions during puberty.
- During early postnatal development.
- During prenatal development.

Sperm production can be altered at any of these developmental stages.

An interference may be a direct one, or may be induced indirectly at either of these stages via an interference with hormonal regulatory processes (figure 2).

In this overview, the discussion will be confined to the possibilities of interfering with sexual hormonal regulations. This does not mean that the direct interference is less significant.

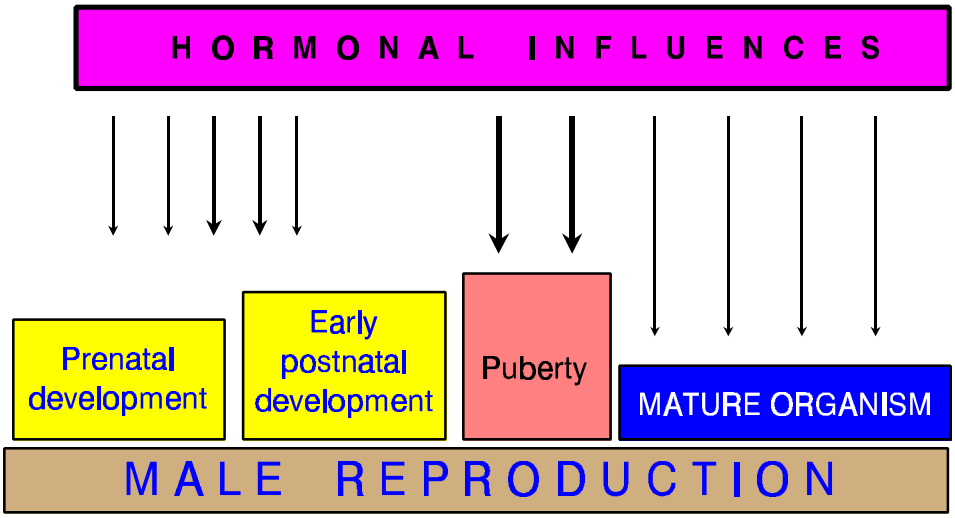

Figure 3: Hormonal influences take place from prenatal development, through puberty to the adult organism. Hormonal influences are essential for the prenatal and early postnatal development of the male sex organs, but apparently also for "imprinting", i.e. irreversibly determining male behavior patterns and functions (*see: figure 5*). A second important influence of hormones occurs during puberty. Of course, adult reproductive functions also critically depend on hormonal regulations.

5. Are there possibilities of interfering with hormonal regulations as a cause of impaired sperm production or altered fertility?

Answer: *Yes. Since sex hormones play a crucial role in regulating sexual functions during almost all periods of development (figure 3), interference with these functions can impair sperm production, fertility, as well as sexual behavior. It is important to recognize that estrogens and androgens, but also antiestrogens and antiandrogens, are capable of interfering with hormonal regulations. Substance-induced effects are naturally not confined to the male, but may also be triggered in the female organism.*

Comment: There is a great deal of information available concerning effects of estrogens and antiestrogens as well as androgens and antiandrogens on general sexual development, along with some data on sperm production. Most of these data come from animal studies performed by the pharmaceutical companies in the development of such substances for therapeutic purposes. For many of these substances there is also ample information on effects in humans. The interested reader is referred to handbook articles in the standard literature.

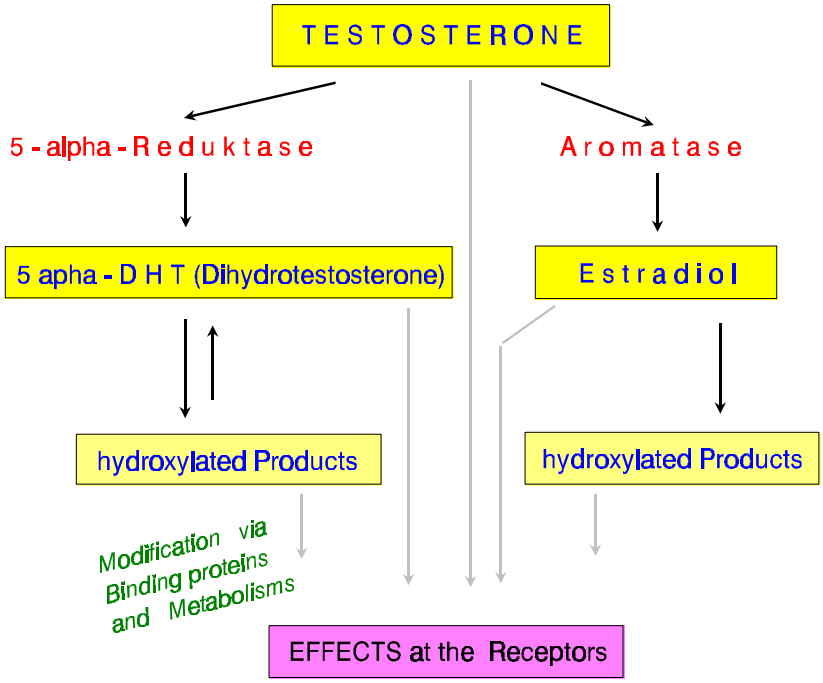

Figure 4: Since androgens can be converted to estrogens within the developing brain, it is difficult to decide which hormone is involved in special reactions. Furthermore, metabolites of testosterone also may be active. Effects on hormonal regulation may also be induced via interference with crucial enzyme reactions, or by interference with binding proteins, competing with the binding to the receptors.

However, effects on hormonal regulations cannot only be induced by excess exposure to hormones or hormone derivatives. Since the normal action of hormones depends on its homoeostasis of the production within the mammalian organism, many enzymic steps are involved in these synthesis pathways. Any substance exhibiting a pronounced effect on these metabolic reactions is bound to disrupt the homoeostasis of this complex hormonal regulatory mechanism (figure 4). Many of such substances interfering with hormone synthesis, including a number of environmental chemicals, are known today from animal studies.

6. Are there environmental substances with affinity to sex hormonal receptors ("ecohormones") exhibiting the potential of interfering with sex hormonal regulation in males?

Answer: *Yes. It is well-known that there are environmental substances exhibiting possible effects on reactions and functions of male reproduction. All these clues come from animal studies. There are almost no data from humans (perhaps some information with DES). Although a possible interference is feasible, it is completely unknown whether the realistic body burdens in the general population reach an order of magnitude sufficient for inducing such a possible effect. Furthermore, it is unknown which of the ecohormones may be realistic candidates.*

Comment: The most important aspect is that a possible hormonal-type of interaction is not confined to an estrogenic potential, although this possibility is given the greatest attention. Androgenic and antiandrogenic as well as antiestrogenic actions may also be of significance, but so far, they have been investigated to a lesser degree. In a recent publication, the antiandrogenic potential of p,p'-DDE, a DDT metabolite with an especially long persistence in the environment, was clearly demonstrated, and a possible significance of such an effect discussed [14]. It is interesting that until then only an estrogenic potential of DDT and its persistent metabolites was taken into consideration.

7. Is it possible to establish a causal relationship between the postulated changes in human semen quality and ecohormones in the environment?

Answer: *No. Much more information must be gathered before such a causal relationship might even be suggested with a reasonable degree of certainty. At present, only speculations are possible, and there is general agreement among experts that a sufficient state of knowledge has not been reached to allow any far-reaching conclusions.*

Comment: As the result of tremendous achievements in chemical analytics, it has been known for a number of years that humans and certain animal species have accumulated body burdens of many environmental substances, including those no longer in use for a considerable length of time (e.g. DDT). Therefore, it is tempting to put the two sets of data (the factual and the assumed decrease in sperm concentration) together, and to speculate on a causal relationship. Such speculations have been offered [15], but confirmation is completely lacking. It represents a speculation on a speculation. Although the problem is generally recognized as such and

additional studies are considered to be essential, scientists knowledgeable in this field have criticized the validity of the data and cautioned against far-reaching conclusions [16,17,18].

With the analytical precision available it is quite clear, even to a layman, that the mere presence of a substance is not identical with it producing an adverse effect. This of course has not prevented the lay sensation press and uncritical "scientists" to be at hand with easy explanations of cause-effect relationships, with titles such as: "An ocean of hormones", or "Unwanted females" [19].

8. Is it possible to recognize interference of ecohormones with male reproductive reactions and functions with the routine studies recommended today (guidelines)?

Answer: *It is only possible to detect very drastic effects. More subtle changes will not be observed with the routine tests (e.g. segment I or II or multigeneration tests in rats).*

Comment: Test methods used for routine assessment of a possible potential of a substance to interfere with male reproduction are largely confined to evaluating fertility, that is the capacity to impregnate a female and to induce a normal pregnancy. Almost all of these studies are performed in rats, although this certainly is not the most suitable species with respect to assessing effects on sperm production. There are methods available to measure sperm quality in adult experimental animals individually over longer periods (e.g. in the rabbit), but these techniques are not used routinely.

Possible substance-induced effects on reproduction and development are currently assessed by experimental designs documented in several guidelines (EU, FDA, OECD, etc.).

There are two different types of procedures to chose from:

- The segment approach (segments I to III), or
- the multigeneration approach (F0 to F2 generation study).

Either of these approaches have advantages and limitations. The duration of the different experimental periods in the segment studies is shorter, possible effects on the various reproductive and developmental stages can be assessed, and specific defects (e.g. malformations) are directly evaluated. The segment II test is performed in two species, but only

in one ("Japanese" protocol for rats) fertility is assessed. Less skill (e.g. on the evaluation of specific structural defects) is required for the multigeneration approach, but because of the long-term study, repetition of the test becomes cumbersome. Often, an overall abnormal development recognized cannot be adequately defined and staged with this latter experimental set-up. Furthermore, with few exceptions, the multigeneration approach is performed in one species only (the rat).

There are four additional peculiarities connected with the experimental designs used that limit the predictive power of both these test approaches:

- With respect to possible effects on male fertility, the rat produces an excess of sperm, and substance-induced reduction of the sperm count to ¼ or even less has little or no effect on male fertility. In humans also no clear-cut correlation of sperm count with fertility exists (except for azoospermia). However, at lower sperm counts longer periods are required to induce pregnancy [20].
- With the usual experimental design only pronounced effects on sexual behavior will lead to a pathological outcome.
- Since the hormonal regulation and the influence of the central nervous system is not identical in rodents and primates, extrapolations of some data obtained in rats to the situation possibly relevant for humans is difficult, if not impossible.
- Since the use of overdoses is called for in the experimental studies (see: most of the guidelines), it is difficult or even impossible to predict possible effects in humans at very low exposures. Many of the animal studies may not be directly relevant to the actual situation in humans. In contrast medicinal products, comparative kinetic data (experimental animal/humans) are not available for environmental substances.

For the reasons mentioned, the standard procedures used to test for possible adverse effects on reproduction and development will not reveal subtle changes in sexual behavior. In special situations it is even doubtful if rodents are the adequate species for drawing conclusions with respect to humans, and studies on non-human primates may be essential for a risk assessment.

It should actually not be necessary to mention that studies on possible "effects" of substances on isolated spermatocytes in vitro (of animals or humans) are largely ineffectual, they might even be rendered nonsensical. Therefore, it would appear to be wasteful to invest time or money in such unsophisticated attempts. This aspect is only mentioned because such attempts have repeatedly

been made over the last years. Every scientist with even minimal knowledge in reproductive toxicology knows that the development of sperm is the important aspect, this is under hormonal control, and occurs largely within a blood-testis barrier. For very good reasons, would no one ever consider "pouring substances on isolated sperm cells" an accepted testing method for possible reproductive toxicity. Data from such "studies" may impress laymen but they are unsuitable for any medical risk assessment, and results of such enterprises merely cause confusion.

9. At which developmental stages may ecohormones trigger an interference with variables of male reproduction, especially sperm production?

Answer: *At almost all developmental stages, from fetal development to the adult state.*

Comment: Effects induced by sexual hormones (androgens and estrogens) may be triggered in mammals at various time periods during pre- and postnatal life:

1. Pre- or perinatal differentiation of sex organs in both sexes.
2. Pre- or perinatal "imprinting" of certain brain functions in both sexes (figure 5).
3. Development of sex organs and sex functions during puberty in both sexes.
4. Maintenance of sexual, reproductive and metabolic functions in the adult of both sexes.
5. Metabolic functions during menopause through estrogen therapy (e.g. with respect to osteoporosis).

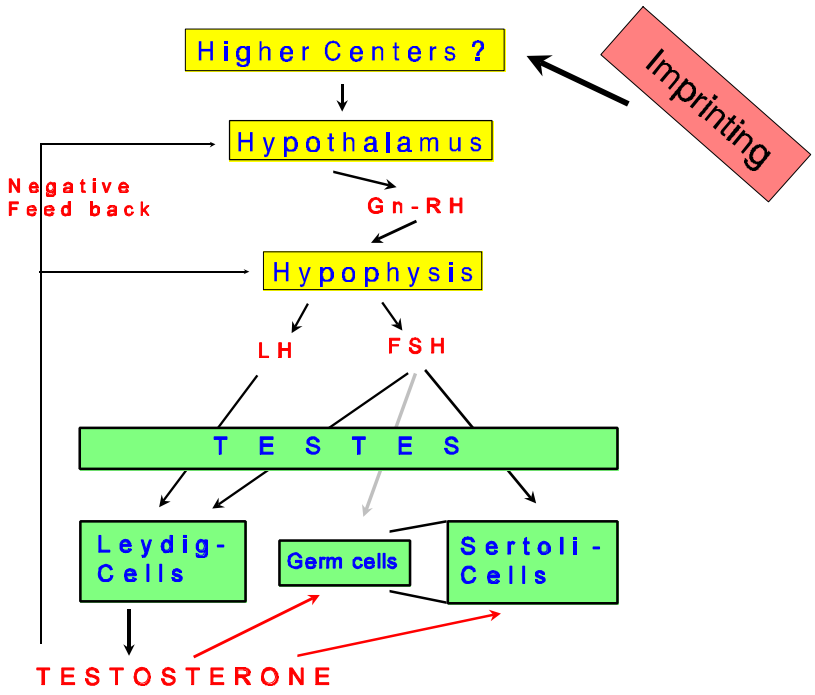

Figure 5: Complex hormonal regulation of testicular function. This regulation may be influenced at all the levels indicated. "Imprinting" seems to occur at the higher brain centers.

We feel that prenatal or early postnatal exposure is of special significance.

When considering sex hormone-induced effects during the pre- and perinatal period, several aspects have to be taken into account:

- All effects are apparently mediated through the typical receptors.
- Androgens as well as estrogens are involved in these processes.
- Androgens may be metabolically converted in the developing brain to estrogens.

Additionally, with respect to possible interference with the development of sexual functions of the brain, the following factors must be considered:

- Pre- and perinatal effects of androgens and estrogens on the developing brain are important for both sexes.
- In contrast to the effects induced by sex hormones at later stages of development, pre- and perinatally-induced effects on the developing brain are often irreversible.
- Indirect factors, such as hormone binding proteins, may play an important modifying role.

10. What information is available on pre-/postnatally-initiated, apparently irreversible, substance-induced effects on sperm quality?

Answer: *Very little and insufficient information. However, there is no doubt that such irreversible effects may be triggered, apparently via different mechanisms, during the pre-/early postnatal development. Neither the sensitive periods nor the substances with a high possible relevance to humans have been sufficiently revealed.*

Comment: It must be stressed that the urgently required information must be obtained from studies at two different levels:

- Studies to reveal possible modes of action that may result in such an effect These studies are essential for developing study designs which have not been established so far.
- Studies to reveal which substances or groups of substances are capable of inducing different types of effects.

Insufficient information is available for either of these issues.

The prenatal development of sexual organs during the late embryonic and fetal period can be disturbed by different substances. This results in a "malformation", possibly connected with concurrent functional defects with respect to fertility. Such defects can also be triggered by hormonal imbalances. This is easy to understand, since the action of androgens is essential for the differentiation of the male organism. Without this influence of androgens, a female phenotype results.

However there is also an effect of androgens on the male sexual development of the **brain**. This is called "imprinting", a term used in different ways in biology. Here it is used to indicate irreversible developmental changes at defined brain centers which determine the male sexual behavior (a failure is discussed as being a possible cause of homosexuality). Apparently, the expression of androgen receptors takes place during this period, and substantial differences remain between males and females in the receptor density in the defined brain regions. Interference with this androgen-dependent development seems to result in feminization of the male organism. It is not quite clear, exactly when this development occurs and how long it lasts, either during the prenatal phase or (more likely) at the early postnatal period. There may be

species differences, like they are known to exist also with respect to other aspects of brain development. While these imprinting effects have been studied rather well in rodents, the significance of these reactions within primates is little understood, especially as far as time period and morphology of the typical changes in the brain are concerned. It is feasible that clear-cut species differences exist, and in order to better understand these developmental processes, more studies in primates are necessary.

The imprinting is apparently mediated through typical sex hormone receptors. It must be stressed that androgens as well as estrogens are involved in this process. Furthermore, it has been demonstrated that androgens may be converted to estrogens in the developing brain, thus making it difficult to put the entire responsibility on the androgens only. Imprinting occurs in both the male and the female organism.

While the described developmental changes certainly have an impact on sexual behavior, they could also influence the hormonal regulations, and thereby be involved in the regulation of sperm production as well. That would indicate that the semen quality of the adult organism could already be influenced by the pre-/perinatal imprinting through androgens.

There is an additional aspect which has been described recently: during the prenatal period the differentiation and the number of Sertoli cells is also determined [21]. Since a close contact with Sertoli cells is essential for sperm maturation, and each Sertoli cell can accommodate only a certain number of sperm, the total daily number of sperm produced may critically depend on the number of Sertoli cells within the testes. Thus, another possibility exists of an irreversible limitation induced prenatally on the sperm quality of the adult organism.

For reasons mentioned we are convinced that a possible interference of a substance with the pre-/ perinatal developmental processes, and the resulting irreversible effects, constitutes an important possibility of obstructing male reproductive development. There are good reasons to believe that interference with these developmental reactions does not lead to "all" or "nothing" phenomena, but instead might result in a graduated response and lesion. The natural variability of these processes may be responsible for the great differences in the sperm concentration between individuals. It should be remembered that there is a poor correlation between sperm count and fertility, and that impregnation can occur also with low sperm counts. Interestingly, in humans there seems to be a clear-cut correlation between the sperm count (or better number of motile sperm) and the time required to impregnate a female [20]. A similar phenomenon can also be observed in rats after treatment with highly toxic doses of TCDD [22].

11. Which studies have been performed and the effects of which substances have been assessed with respect to pre-/early postnatal induction of abnormalities in reproductive performance?

Answer: *Rather extensive studies have been performed on sexual performance and sexual behavior after pre-/early postnatal exposure of rats (Peterson and co-workers). These studies were performed with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). While these studies supplied ample information on possible effects on a variety of endpoints, the information is still limited because:*

No NOAEL could be established;

Several of the data apparently have neither been confirmed at the same lab or elsewhere;

Apparently doses in the order of magnitude around 1 µg TCDD/kg body wt were required

for a clear-cut effect. This dose is close to the dose initiating effects in the adult rat;

It has not been established that an "estrogenic" action of TCDD is responsible for the effects reported;

The significance of some of the variables tested for the situation relevant to humans remains quite unclear.

No other substance has been tested in a similar way.

Table 3: Changes reported by **R.E. PETERSON** and co-workers in offspring of rats treated with a single dose of TCDD on day 15 of pregnancy [paper # 1, Mably et al. 1992a].

Dose (ng/kg bw)	Effect	Period	Change	Reference
1,000	Testosterone/blood plasma, males 1	Days 18/19 prenatally	**Reduction**	
	Testosterone/blood plasma, males 1	Day 2 postnatally	**Reduction**	
	Body weight, dams 1	all pregnancy	no	
	Gestational index 1	mating + pregnancy	no	

1	Litter size	birth	no
1,000	Number of viable newborn	birth	**Reduction**
1			
400		birth	no
1			
160		birth	no
1			
64		birth	no
1			
1,000	4-day newborn survival index	postnatal development	no
1			
1,000	21-day newborn survival index	postnatal development	no
1			
1,000	Postnatal body weight gain	postnatal development	**Reduction**
1			
400		postnatal development	**Reduction**
1			
160		postnatal development	no
1			
64		postnatal development	no
1			
1,000	Pinna detachment	postnatal development	no
1			
1,000	Incisor eruption	postnatal development	no
1			
1,000	Eye opening	postnatal development	Reduction
1			
400		postnatal development	no (?)
1			
160		postnatal development	no
1			
1,000	Testes descent	postnatal development	Retardation
1			
400		postnatal development	Retardation
1			
160		postnatal development	Retardation
1			
64		postnatal development	no
1			
1,000	Anogenital distance, males	postnatal development	Reduction
1			
400		postnatal development	Reduction
1			

160	1		postnatal development	Reduction
64	1		postnatal development	no

Table 4:

Changes reported by **R.E. PETERSON** et al. in offspring of rats treated with a single dose of TCDD on day 15 of pregnancy [paper # 1 Mably et al. 1992a].

Postnatal day of evaluation	Dose of TCDD on day 15 of pregnancy (ng/kg bw)			
	64	160	400	1,000
Seminal vesicle weights:				
32	O	ê	ê	ê
49	O	O	ê	ê
63	O	ê	ê	ê
120	O	O	O	O
Ventral prostate weights:				
32	ê	ê	ê	ê
49	O	O	ê	ê
63	O	ê	ê	ê
120	O	O	O	ê
Blood plasma LH concentration:				
32	O	O	O	ê
49	O	O	O	O
63	O	O	O	O
120	O	O	O	O

Comment: Publications of extensive studies on possibly irreversible alterations in sexual functions and behavior which were induced **pre-/perinatally** and manifested itself at sexual maturity were only presented by Peterson and co-workers [23-27]. In these studies 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was used as the toxic principle.

It should be stressed that all the data available at present are, for many reasons, quite insufficient to serve as a basis for a medical risk assessment with relevance to humans. Some of

the reasons will be discussed later. It is the aim of the following discussion to mention the variables which can be evaluated.

Since the studies of Peterson and co-workers attracted most of the attention and may be taken as an example of possible effects on male reproductive components and functions induced during the pre-/perinatal period, these data shall be presented and discussed here in some detail. The first three reports published in 1992 were apparently data from the same experiment. The authors performed an array of tests in the male offspring exposed *in utero* to TCDD. Since the elimination half-life of TCDD in rats is long (about 3 weeks), additional exposure of the offspring to TCDD during lactation is likely. However, it should be remembered that secretion via the milk is a very effective way of eliminating this substance in the rat [28], and the maternal body burden and thereby the concentration in the milk will decline rapidly.

Table 5:

Changes reported by **R.E. PETERSON** et al. in offspring of rats treated with a single dose of TCDD on day 15 of pregnancy [paper # 2 Mably et al. 1992b].

Postnatal day of evaluation	Dose of TCDD on day 15 of pregnancy (ng/kg bw)			
	64	160	400	1,000
Number of intromissions preceding ejaculation:				
56-63	O	O	O	O
70-77	O	O	O	é
112-119	O	O	O	O
Number of mounts preceding ejaculation:				
56-63	O	O	O	O
70-77	O	é	O	é
112-119	é	é	é	é
Copulatory rate:				
56-63	O	ê	ê	ê
70-77	O	ê	ê	ê

112-119	O	ê	ê	ê
	Postejaculatory interval:			
56-63	O	O	O	é
70-77	O	O	O	é
112-119	O	O	é	é

For this reason, it is impossible to judge which doses or tissue concentrations have been established during the studies performed by Peterson et al.

Following the single oral administration of 1 g TCDD/kg body wt on day 15 of pregnancy (the highest dose used), a considerable number of abnormalities in sexual behavior was observed in the male offspring. It would be a misinterpretation to assume that the effects must have been triggered on the day of gestation the substance was administered. From the schematic compilation in tables 3 to 7 it can be seen that some of the effects were already reported at much lower doses.

The lowest dose used was 64 ng TCDD/kg body wt, and a few effects were reported for the group treated with this dose.

Table 6:

Changes reported by **R.E. PETERSON** et al. in offspring of rats treated with a single dose of TCDD on day 15 of pregnancy [paper # 3 Mably et al. 1992c].

Postnatal day of evaluation	Dose of TCDD on day 15 of pregnancy (ng/kg bw)			
	64	160	400	1,000
	Testes weights:			
32	ê	O	ê	ê
49	O	O	O	ê
63	O	O	ê	O
120	O	O	O	O

Right epididymis weight:				
32	O	ê	ê	ê
49	ê	ê	ê	ê
63	O	ê	ê	ê
120	ê	ê	ê	ê
Right cauda epididymis weight:				
32	O	ê	ê	ê
49	O	O	ê	ê
63	ê	ê	ê	ê
120	ê	ê	ê	ê
Daily sperm production, right testes:				
49	O	ê	ê	ê
63	ê	ê	ê	ê
120	ê	ê	ê	ê
Right cauda epididymal sperm numbers:				
63	ê	ê	ê	ê
120	ê	ê	ê	ê

As should be expected, there was no impairment of fertility in these animals. This is not too surprising, since rats have a large surplus of sperm, and reduction in sperm number must be drastic in order to impair fertility.

Although the studies of Peterson and co-workers are the most extensive ever reported on this subject, they still suffer from a number of inadequacies, and several of the effects reported for the low dose levels (< 700 ng TCDD/kg body wt) apparently could later not be confirmed at the same laboratory [27,29], and the authors used considerably higher doses (1 µg TCDD/kg body weight) in the subsequent studies.

Table 6a:

Changes reported by **R.E. PETERSON** et al. in offspring of rats treated with a single dose of TCDD on day 15 of pregnancy [paper # 3 Mably et al. 1992c].

Postnatal day	Dose of TCDD on day 15 of Pregnancy (ng/kg bw)

of evaluation	64	160	400	1,000
CONTINUATION				
Sperm motility:				
63	O	O	O	**?**
120	O	O	O	**?**
Sperm morphology:				
63	O	O	O	O
120	O	O	O	O
Ratio of leptotene spermatocytes to Sertoli cells in testes:				
49				O
63				O
120				O
Plasma FSH concentration:				
32	**ê**	O	**ê**	**ê**
49	O	O	O	O
63	O	O	O	O
120	O	O	O	O
Reproductive outcome (70-day-old exposed males with control females):				
Fertility index	O	O	O	**?**
Gestation index	O	O	O	O
Litter size	O	O	O	O
Live birth index	O	O	O	O
21-day postnatal survival	O	O	O	O
Pup body weight, day 21	O	O	O	**?**

Table 7:

Changes reported by R.E. PETERSON et al. in offspring of rats treated with a single dose of TCDD on day 15 of pregnancy [paper # 4 Bjerke et al. 1994].

Dose of TCDD on day 15 of pregnancy = **700** ng/kg bw

Mean body weight and mortality of male offspring:
*Body weight was **decreased** (to 89-92%) between birth and day 7 postnatally.*
*Offspring mortality was **7%** in controls, **23%** in exposed offspring.*

Relative anogenital distance:
***No significant difference** to controls.*

Age at testes descent: Age at preputial separation: Testis wt on day 63:
Were slightly (statistically significant) changed.
*Difference of mean values of exposed to controls: **4%, 4%, 7%**.*

Ventral prostate weight after castration and testosterone substitution
***Significant difference** to controls.*
*DNA content: **no significant difference** to controls.*

Seminal vesicle weight after castration and substitution
***No difference** to controls.*

Testosterone after castration and substitution
***No difference** to controls.*

LH after castration and testosterone substitution
***No difference** to controls.*

Ventral prostate testosterone and 5 € DHT content after castration and testosterone substitution
***No significant difference** to controls.*

Conclusions from these studies:

TCDD exposure does not result in a generalized inhibition of imprinting.

It appears that responsiveness of cells to hormones

and/or growth factors is altered.

Many of the results reported earlier ***could neither be confirmed***

at the same lab nor elsewhere.

These doses are well-known to produce already first signs of general toxicity on peri-/early postnatal development of rats. It appears that prostate weight, but not that of the seminal vesicle is a sensitive variable. This organ weight is reported to be decreased, even in the absence of a clear-cut change in hormonal concentrations [29]. In all these papers, the interpretation of the data is hampered by the fact that only mean and SE values are given in the presentations. Furthermore, the higher doses induce clear-cut signs of general toxicity in the offspring (weight retardation, increased mortality, etc.).

Explicit effects were demonstrated with the single dose of 700 ng or 1,000 ng TCDD/kg body weight. This dose level is not too far away from that required to induce an effect on sperm production in the adult animal [30]. In this respect it seems important to remember that TCDD can typically interfere with sperm maturation and production also in the adult rat, especially at high dose levels [22,30,31,32]. There is no indication that this effect is caused by alterations in the sex hormone regulation. Light and electronmicroscopic evaluations revealed a disturbance of intercellular contacts between Sertoli cells and spermatogonia. Since in all these studies from various laboratories only TCDD was tested until now, it is completely unknown whether "TE-factors" can also be applied to this type of adverse action when assessing a possible potency of other PCDD/PCDF congeners or of PCBs. Since sex hormone-dependent development and cerebral imprinting occurs in both sexes, from the available data a statement on the possible susceptibility of female development cannot be made.

Similar effects may also be induced by other substances, e.g. DDT and metabolites or €-hexachlorocyclohexane (Lindane®). However, no published data are available for these substances.

12. Further research needs

The results discussed here represent only a very early starting point for investigations in this area.

The problems should not be underestimated, and extensive studies must be initiated soon. This will be no easy task, since no "simple" methods are at hand for this purpose and considerable experience and expertise in the field of reproductive toxicology are necessary. Studies to be performed are almost exclusively complex long-term studies, they only warrant financing if combined with comprehensive kinetic investigations, and they cannot be confined to

investigating rodents. It will be necessary to evaluate whether primates respond the same way as rats (which is not very likely), and again, such studies can only be performed at very few institutions having both the experience with non-human primates and with reproductive toxicology. It would be best to create a body of experts to decide where which studies can be performed. This however, will only be viable if the necessary funds are provided. In order to avoid adding to the confusion, it would be advisable not to finance studies of limited scope and quality. It is indeed puzzling that so far, Germany has completely missed any opportunity to institute large-scale studies which are of extreme importance for generations to come. The hope remains that in the near future German agencies (e.g. the BMBF and the Kernforschungszentrum Karlsruhe) will be so far-sighted and prudent to utilize the splendid resources available in our country for initiating studies which would help to solve these problems.

13. Conclusions

There are a number of substances in our environment which exhibit properties of sex hormones. These chemicals may conveniently be called: "ecohormones". There are a number of indications from studies of wildlife and other ecosystems that these substances may cause alterations in animals [33]. Which substances are specifically responsible has not been established in most cases.

Although it is known that certain ecohormones at overdoses perhaps induce alterations in the male reproductive system of experimental animals, many of the underlyingmechanisms are still poorly understood. Furthermore, it is not known which types of substances will pose a possible problem under *in vivo* conditions and when considering the realistic concentrations present today.

At present the scientific community cannot arrive at any conclusions on possible interactions of "ecohormones" with the male sex hormonal regulatory system at the realistic and existing body burdens of humans. Any association of such ecohormones with a possible (but not at all proven) decline in human sperm concentration are still purely speculative. However, possible effects of such ecohormones on humans, especially on certain populations with higher body burdens and possibly at risk, cannot be ruled out. Therefore, extensive studies, both experimental and epidemiological, will be required to solve these problems.

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Studies on the Influence of Dioxins and other Chlorinated Hydrocarbons on Human Fertility

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Introduction

Recently reports about a reduction of human fertility in western countries have become more and more frequent. Several phenomena such as social drugs, stress, and environmental contaminants have been blamed as causal. Among the latter, persistent organochlorine compounds, especially "dioxins" and polychlorinated biphenyls (PCB), but also household insecticides of the pyrethroid group have come into the centre of attention.

"Dioxins" is the trivial name for two ubiquitous groups of aromatic polycyclic halogenated hydrocarbons. These are **p**oly**c**hlorinated **d**ibenzo-para-**d**ioxins (PCDD) and polychlorinated dibenzofurans (PCDF). Some members of these groups are highly toxic. The compounds are not easily degraded in the environment and are therefore very persistent. Their distinct lipophilic character facilitates their accumulation in the food chain and in human tissue. Little is known about their reproductive toxicity in humans.

Experiments with human and similar cell systems were carried out to assess potential risks to reproductive functions arising from these compounds. Of particular interest were those functions that are of particular importance for human fertility. Sperm motility, embryonic preimplantation development and hormone production of trophoblast cells under the influence of dioxins and some other halogenated hydrocarbons were studied.

Methods

Fresh human ejaculates were density fractionated, and the highly motile sperm cells thus obtained were incubated in various dioxin-containing or control media. Incubated spermatozoa were sampled at regular intervals over a period of 60 hours, and their motility was determined using automated motility analysis. Similar experiments were also carried out in the presence of various PCB compounds ("congeners") and pyrethroid compounds.

Two-cell mouse embryos were incubated in dioxin-containing or control media for a period of 72 hours and studied under the microscope for the influence of various dioxin congeners on embryonic development.
Human chorionic carcinoma cells were incubated in dioxin-containing or control media to study the effect of highly toxic and low-toxic dioxin congeners on the proliferation and hormone secretion of trophoblast cells. Cell proliferation was determined microscopically and by measuring the radio thymidine incorporation. The concentrations of human chorionic gonadotropin (hCG), progesterone and estradiol in the culture supernatants were measured by radioimmunoassays.

Results

In-vitro motility of human **sperm cells** could neither be influenced by the low-toxic 1,2,3,4-**t**etra**c**hloro**d**ibenzo-p-**d**ioxin (1,2,3,4-TCDD) nor by the highly toxic isomer 2,3,7,8-TCDD. Similarly, highly toxic and low-toxic PCB congeners (PCB 77 or PCB 101) and the pyrethroids cyfluthrine and permethrine did not influence sperm motility under the selected conditions chosen. (Fig. 1).

Mouse preimplantation embryos were used as a model of early human embryonic development. Several highly or negligibly toxic PCDD congeners were shown to cause a significant retardation in embryonic preimplantation development. Further, the percentage of degenerated embryos was elevated under the influence of PCDD. While only 18% of embryos were classified as degenerated in the control group, 1,2,3,4-TCDD at 50 ng/ml lead to a rise to 51% degenerated forms ($p < 0.001$) (Fig. 2).
Human **chorionic carcinoma cells** served as an in-vitro model of early periimplantation cytotrophoblasts. Dosage-dependent cytotoxicity and impairment of hCG, progesterone, and estradiol secretion were observed under the influence of 2,3,7,8-TCDD and 1,2,3,4-TCDD (see Fig. 3 for hCG). Subtoxic concentrations of both substances caused a significant increase in estradiol production.

Conclusions

The experiments described showed that PCDD do have a potential of interfering with fertility Concentrations required to bring about toxic effects in vitro (about 10^{-9} molar) were at least 1,000 times higher than the present contamination of human gestational tissue and the background concentations of other human tissues and body fluids.

Such high concentrations, however, were reached in vivo in the aftermath of the Seveso accident (1976). The persons affected survived and are now in their reproductive age. As concentrations in the tissue are still very high, the findings presented here could be of importance for these persons.

Epidemiological studies of population groups that were accidentally contaminated with dioxins and furans have not yet proved of a generally teratogenic or deleterious effect of PCDD/PCDF to human reproduction.

However, the findings presented in this paper stress the need for further research into subtle effects of low concentrations of PCDD/F on human reproduction. In this respect the modulation of steroid hormone secretion could be of particular interest.

Fig. 1: Incubation of sperm cells in PCB-containing medium does not influence motility (PCB 77: 0.2 µg/ml; PCB 101: 0.03 µg/ml; similar effect observed up to 6.6 µg/ml PCB 77) mean values, standard deviation for PCB 77, n=9

Fig. 2: Incubation of CB6F1 mouse embryos in a dioxin-containing medium: Increase in percentage of forms, reduction in hatched blastocysts
1,2,3,4-TCDD: 52 ng/ml; 1,2,3,4,7,8-HexaCDD: 5.6 ng/ml; n > 150/group

Fig. 3: Incubation of human JAR chorionic carcinoma cells in culture TCDD-containing medium : dose-dependent influence on hCG concentration in the medium
mean values, standard deviation, n=18;
* asterisk: significant difference ($p < 0.05$) from controls in a Scheffé test

Endocrine Disruptors and the Human Female Reproductive System

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In the past few years, in our laboratory we have mainly dealt with the role of eicosanoids in human reproduction. Today's topic is therefore not new to us as processes regulated by hormones are not only influenced by intrinsic but also by extrinsic stimuli. We became interested, in this connection, in the question whether foreign substances that are present in the environment can interact with substances that determine human reproductive functions. I would like to focus here on discussing the relevance of this topic for the female reproductive system, and mechanisms of action. And finally I would like to present two of our own projects on this topic.
Reproductive organs are a major target of toxic chemicals. This has been described in some detail in the two papers presented before.

The female reproductive system has a much more complex structure than the male one. Not only the maturation of gametes, but also their fertilization and implantation as well as the carriage of the fetus take place here and depend on a balanced system of interaction. The first table shows in vivo findings about the reproductive toxicity of some chemicals. These aspects were discussed earlier in some detail. Such negative effects on humans and wild animals have been described for the DES syndrome and after large-scale CHC pollution in the United States. In long-term animal experiments with dioxins impaired fertility and alterations in sexual behaviour of male rats and induce an increased rate of endometriosis in female rhesus monkeys have been observed.

Table 1 : In - vivo findings

1. DES syndrome (before 1972) : Increased rate of clear cell adenocarcinoma of the vagina and reproductive dysfunctions in daughters of person treated during pregnancy

2. DDT pollution of "Lake Apopka"/Florida (1980): 90% reduced birth rate of alligators , increased rate of malformation of male genitals

3. Pollution of the Great Lakes with DDT, PCB and dioxins (before 1990):
Fertility disorders and high embryo mortality in wild species (above all, sea eagles and other birds, fish)

4. Toxic effects of TCDD on the reproductivity of male rats after perinatal exposur e (single application of 64 ng/kg body weight to the mother animals; Mably et al. 1992)

5. Increased rate of endometriosis in a long-term study of TCDD in rhesus monkey s (Rier et al. 1993).

In this connection it is also important to note that the reproductive system showed a more sensitive response to foreign substances than other organs in most animal experiments. Such observations on PCB effects on laboratory animals were summarized, for example, by Golub et al. (1). This data shows that prenatal development is particularly vulnerable to the activity of harmful substances. Fertility, however, can be disturbed as well by comparatively low doses of PCB.

Are hormonally active chemicals in the environment a risk for human reproduction? We think this cannot be assessed with any certainty at the moment. From the findings quoted we know, on the one hand, that many xenobiotics are capable of interfering with reproduction. Many studies including our own upfront experiments have proved the internal exposure of reproductive organs to organochlorine substances. In persons with an average exposure, these substances were found at low concentrations in the follicular fluid, the cervical mucus, the myometrium, endometrium and seminal plasma. It is important to note that, based on wet weight, lipophilic organochlorine substances accumulate in the endometrium rather than in the myometrium. This can be explained by the higher lipid content of the endometrium. This finding is of great importance because vital and very sensitive biological processes take place in the endometrium, for example, implantation and early embryonal development. Contamination of the endometrium with xenobiotics should therefore be taken very seriously in risk assessment.

Mr Hanf has explained the target organs in the female reproductive system. I would like to make a few remarks about the mechanisms of action, protective mechanisms, and substances that show endocrine activity.

First, mechanisms of action. A great number of mechanisms has been described that enable hormonally active substances to influence reproductive processes. Let us just briefly take a look at the basic communication mechanisms of the hormonal system. In the classic endocrine system, a gland secretes a hormone that is transported by the circulating blood to an often remote target organ. Auto- and paracrine regulation takes place at organ and tissue level. The signal molecule has a direct retroactive effect on the cell where it was formed or on neighbouring cells.

The ability of chemicals in the environment to mimic the hormonal effects of endogenous hormones is an important mechanism of action. Environmental chemicals with estrogenic activity are in the centre of interest here (2). Estrogens are certainly the most important female sexual

steroids. They simulate nearly all processes of development and growth. Their activity must therefore be balanced by endogenous antagonists such as the gestagens in the endometrium.

Other xenobiotics can directly counteract endogenous hormones. They either block hormone receptors or impair them by down regulation. Then there are substances that indirectly interfere with hormonal balance. These are substances that intervene in the biosynthesis or degradation of hormones.

We know little about interaction of environmental chemicals with autocrine or paracrine regulatory mechanisms. An important mechanism of action, though insufficiently studied, is the induction of cytochrome P-450 (CYPIA1) by dioxins and PCBs. It is also known that CYPIA1 and P-450 isoforms can metabolize arachidonic acid (3). P-450 induction triggers alterations in the arachidonic acid cascade which is an important mediator system (4,5). Such effects were induced in vitro using a chicken embryo model. Another type of interaction of harmful substances and autocrine or paracrine mechanisms is the induction of hormone receptors. Table 2 describes some essential substances that are known to have an endocrine effect. These include environmental estrogens, i.e. harmful substances that imitate the activity of the natural sexual hormone 17€ -estradiol (estrogenicity is given here with reference to estradiol) based on the E-Screen assay (6).

Table 2 : Estrogenes in the Enviroment

diethylstilbestrol	(10)	dieldrin	(0,000001)
zearalenone	(0,01)	endosulfan	(0,000001)
coumestrol	(0,00001)	toxaphene	(0,000001)
4-nonyl phenol	(0,00001)	methoxychlor	
o,p'-DDT	(0,000001)	[17€ -estradiol	(1)]

Other endocrinically active chemicals in the environment

PCDD/PCDF	hexachlorobenzene
PCB/BB	chlordane
triazine herbicides	lindane
phthalates	Hg, Pb, Cd

You will find that most harmful substances in the environment are very weak estrogens, except for diethylstilbestrol (DES), a synthetic estrogen that is by ten times more efficient than natural

estradiol. Not only synthetic xenobiotics are a problem in this context but also natural substances such as zearalenone, a mycotoxin, and coumestrol, a phytoestrogen.

But there are other chemicals in the environment besides estrogens that interfere with the hormonal balance by other mechanisms. Dioxins and furanes as well as coplanar PCBs are capable of disturbing the arachidonic acid metabolism through cytochrome P-450 Antiestrogenic effects were also described for TCDD, the best known dioxin (7). But some PCB congeners and PCB metabolites have an estrogenic effect (8). Triazine herbicides and phthalates, on the contrary, intervene in the biosynthesis and decomposition of steroid hormones (9). Some chlorinated insecticides are also capable of disturbing the steroid balance. I will not go into effects of heavy metals on the hormonal system here.

We will also have to discuss protective mechanisms that the system uses to fend off undesired activity of harmful substances. The body contains a great number of estrogenically and anti-estrogenically acting substances, one might speak of an "ocean of estrogens and anti-estrogens". So the system has the potential to compensate undesired estrogenic loads. But there are some open questions here to which I will return in a minute. Furthermore, the system can in many cases decompose endocrinically active substances (e.g. phthalates) by metabolic processes.

Let me make a few remarks about the problem of measuring estrogenicity. The classic proof of estrogenic activity is obtained by measuring the uterotropic activity of a substance in the rat. An estrogenic substance will trigger a certain growth of the uterus depending on its efficiency and dosage. The animals have to be killed to weigh their uteri. This method can therefore not be used as a general screening procedure but is accepted as gold standard. Another bioassay was developed by Ms Soto (U.S.A.) based on the estrogen-sensitive MCF7 breast cancer cell line (6). What is measured here is cell proliferation as a function of estrogenicity of a substance. A third classic detection method is the measurement of the amount of 17 -estradiol displaced from its receptor by estrogenically active substances (8). In a newly developed method, an estrogen-sensitive gene product is coupled with chloramphenicol transferase as reporter gene.

Some important questions will have to be answered before risk assessment can be carried out on a well-founded basis:

1. The lack of data about concentrations in target tissues (above all, for organochlorine compounds) should be overcome.

2. The question what problems continuous unphysiological low-level stimuli caused by xenobiotics pose for the prevailing pulsatile hormonal secretion should be studied in detail.

3. There should be a closer study of the bioavailability of hormonally active xenobiotics. While a considerable percentage of sexual steroids is bound to binding proteins and is therefore not bioavailable, xenoestrogens are bound to proteins only to a limited extent. Their higher bioavailability might compensate for much of its lower estrogenic effect. It should also be taken into account in this respect that the blood may contain environmental chemicals at higher concentrations than endogenous hormones.

4. The effects of chemicals in the environment on paracrine and autocrine regulation should be given much more attention by researchers.

5. It should be examined to what extent metabolites of non-endocrine xenobiotics can have an endocrine effect.

6. Moreover, it should be examined whether persistent xenobiotics may delay the switching off of a hormonal signal, thus causing a protracted effect.

Concludingly I would like to say that with our present knowledge we have not yet been able to prove a causal connection between contamination with xenobiotics and dysfunctions of human reproductive organs. But based on the above-mentioned in-vivo findings in wild animals, animal experiments and in-vitro findings, we think that the theoretical risk is too great to be ignored. We cannot exclude even from a system theoretical point of view that minor causes can result in pathogenic effects on the highly complex and synchronized control circuits of the reproductive system.

Our own projects

a) Studies of the Effect of Xenoestrogens on Endometrial Functions in Humans (sponsored by PUG, Karlsruhe Research Centre)
Initial funding to launch the project was guaranteed for one year. There are two parts of the project for which funds were granted: First, we will compile a list of substances from data provided in the relevant literature that will group xenoestrogens according to their estrogenicity and their occurrence in Germany. We intend to find out in this way which xenobiotics, based on the relevant literature, are of interest at all for us. This list of substances will be the basis for studying "internal exposure" of endometrial biopsies to the relevant xenoestrogens. If these exposure studies reveal a risk for the endometrial function, we will apply for funds to make activity experiments on human endometrial cultures as of 1996. We started the work on our list of substances in february. We first determined all substances that are associated with estrogenic or anti-estrogenic activity in the relevant databases. I would like to present this preliminary list to you as it includes a great number of substances. We will now check these substances for valid hormonal effects and try to find exposure data for Germany and data on estrogenicity for the remaining substances.

b) Dioxins, Furanes, Polychlorinated Biphenyls and Human Reproduction
Our application for this study was positively evaluated in December 1994, and if the Federal Ministry for Research and Technology gives its approval we will launch the project in 1996 We will study selected PCB congeners and 2,3,7,8-TCDD to find out whether these substances affect the paracrine and autocrine regulation of reproduction-specific cells and tissues. We will measure various functional parameters in granulosa cells, endometrial cells, peritoneal macrophages, the placenta, and the decidua. It is the purpose of our studies to

find out which of these tissues are sensitive to PCBs and dioxins and which biomarkers produce detectable effects.

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Data from Animal Experiments and Epidemiological Data on Tumorigenicity of Estradiol Valerate and Ethinyl Estradiol

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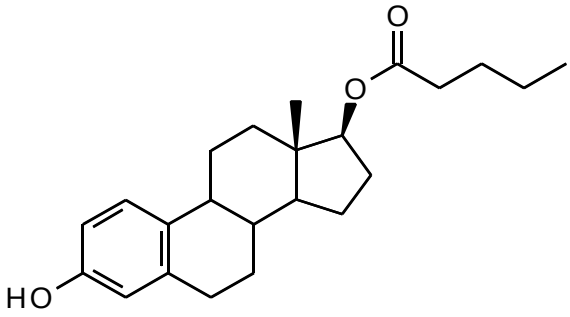
Introduction

The problem of whether estrogenic substances in the environment might play a causal role regarding tumorigenicity in humans, especially with regard to higher incidence in breast cancer in women is the subject of public debate (1). The term “environmental estrogens“ covers a multitude of chemicals of both natural (from plants and animals) and purely synthetic origin with very different chemical structures (1,2). Furthermore, the postulated estrogenic modes of action of many of these chemicals and the induced effects seem not to have been sufficiently tested in respect of their pharmacological properties (2). This makes the discussion on potential causal interrelations between the actual estrogenic function of chemicals (or mixtures of chemicals) and their, in some cases only suspected, tumorigenic potential even more difficult.

In this context data from animal experiments on the tumorigenicity of the steroid estrogens estradiol valerate and ethinyl estradiol, both relatively well characterized with respect to their pharmacological properties, will be presented in the following. Both estrogens are known to be very reactive in animals and humans and have been widely applied for therapeutical purposes in women for decades. This long experience in humans enables us to assess the extent to which the results obtained in annual experiments can be extrapolated to the effects observed in humans.

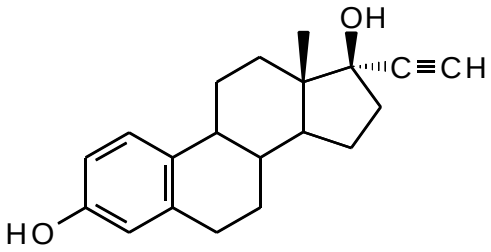
With regard to structure, estradiol valerate is a fatty acid ester of the natural sex hormone 17ß-estradiol (Fig. 1). When estradiol valerate is taken up by mammals the ester bond is split, and 17ß-estradiol is released as estrogenic component. Estradiol valerate is used for therapeutical purposes as a single substance or in combination with gestagens to treat complaints in the climacteric period. The therapeutical dosage of the estrogen for oral application is usually 2 mg estradiol valerate/woman/day.

Figure 1:
Chemical structure of estradiol valerate



In ethinyl estradiol the steroidal main structure of 17ß-estradiol is connected with an ethinyl group (Fig. 2). In contrast to estradiol valerate ethinyl estradiol acts directly without further metabolic transformation. Since the ethinyl group leads to an increase of metabolic stability after oral administration of the latter substance, bioavailability is higher than that of the unchanged 17ß-estradiol. Ethinyl estradiol is mainly applied as the estrogenic component of oral hormonal contraceptives in combination with various gestagens. In modern, low dosed oral contraceptives the daily dose is 20 to 50 µg/woman over the 21-day administration/treatment period.

Figure 2:
Chemical structure of ethinyl estradiol



Data from animal experiments on the tumorigenicity of estradiol valerate and ethinyl estradiol

In the scope of a toxicological test program aiming at a risk assessment of long-term application in humans, long-term animal experiments have been performed with the two estrogens to obtain information about their potential to influence the incidence of tumors and their growth. Especially ethinyl estradiol, which is commonly used as a component of oral contraceptives for long-term application in healthy young women, has been investigated in a great number of toxicological

studies with animals, both as single substance and in combination with gestagens. Table 1 shows the design of some tumorigenicity studies with estradiol valerate and ethinyl estradiol, respectively, administered as single substance (i.e. without simultaneous application of gestagens).

According to common practice in the sixties, the dosages were selected as multiples of the intended human dosage related to the body weight. With regard to the common dosages of 2 mg estradiol valerate/woman (corresponding to approx. 0.04 mg/kg body weight) and maximally 50 µg ethinyl estradiol/woman (corresponding to approx. 1 µg/kg body weight) the maximum dosage for rodents was at least 100 times higher than the human dosage; for dogs a dosage 25 times higher and for monkeys a dosage 50 times higher were administered as the highest dosages according to the guidelines of the FDA (3). In view of the present knowledge it should be pointed out that in the described concept of “safety margins“ between the dosages in animal models and the human dosage the clear-cut pharmacokinetic differences, especially the lower oral bioavailability and the higher rate of metabolism of estrogens in animals versus humans, were not considered (5).

Table 1: Study design of tumorigenicity studies with estradiol valerate and ethinyl estradiol, respectively

Animal species	Number and sex	Route	Dosage (mg/kg/d)	Duration
Study with estradiol valerate				
rat/ Charles River CD	20 m/30 f 20 m/30 f 20 m/30 f 20 m/30 f	oral (food)	0 1.2 3.6 12.0	90 weeks
Studies with ethinyl estradiol				
mouse/ CF-LP	40 m/40 f 40 m/40 f 40 m/40 f 40 m/40 f	oral (food)	0 0.005 0.030 0.200 *	80 weeks
rat/ Holtzmann albi.	100 m/100 f 50 m/50 f 50 m/50 f	oral (food)	0 approx. 0.008 approx. 0.08	105 weeks
dog/ Beagle	12 f 12 f 12 f	oral (capsule)	0 0.01 0.025	7 years **
monkey/ Macaca mulatta	16 f 16 f 16 f 16 f	oral (food or gavage)	0 0.002 0.01 0.05	10 years **

m male animals
f female animals
* dose reduced to 0.09 mg/kg/day in week 51
** cyclical treatment: 21 d application and 7 d without application per cycle

Taking into account the pharmacokinetic differences it has to be assumed that the real systemic load of estrogens in the whole organism in the above mentioned animal studies was probably still below the human systemic load. However, comparative investigations on endocrinology of sex steroids in test animals and in humans revealed that there are great differences regarding endogenous estrogen levels, endocrine regulation mechanisms as well as sensitivity of target organs in different animal species (5). Therefore, considering also pharmacokinetic data, the concept of "safety margins" is generally not suitable for testing sex steroids on the one hand or in particular for the toxicological characterization of estrogens on the other hand. Some aspects of

fundamental differences between animal models and humans will be briefly discussed in the following.

Results of animal studies and discussion of relevance for humans

The organs for which an increased tumor rate was observed in the above mentioned studies are given in table 2. Further target organs and tissues for potential tumorigenic effects after application of estrogens in animal models described in literature are the vagina, testicles, lymphatic organs and bones of mice, ovaries, the uterus and cervix of rats and the kidney of hamsters (4,6).

The most obvious effect after administration of the two estrogens with regard to tumor development was a higher incidence of pituitary adenoma in both rodent species. In rodents a relatively high rate of pituitary tumors is generally observed in untreated animals as well. In the above mentioned studies pituitary adenomas were found in 10 to 20 % of the male control animals and in 30 to 40 % of the female control animals. The high susceptibility of rodents with regard to the induction of pituitary tumors by estrogens is due to their particular endocrine regulation (see ref. 5 and 6). Therefore, the described effect on the pituitary has neither been observed nor is to be expected for other animal species (dog and monkey) or humans following therapeutical dosages usually applied.

Table 2: Tumorigenic effects of ethinyl estradiol (EE2) and estradiol valerate (EV) in different animal species.

Species	**Rat**	**Mouse**	**Rat**	**Dog**	**Monkey**
Test Substances	**EV**	**EE2**	**EE2**	**EE2**	**EE2**
Organ/Tumor					
pituitary/adenoma	€ m + f	€ m + f	€ m + f	-	-
mammary gland/all types	€ m	-	-	-	-
liver/nodular hyperplasias	-	-	€ m + f	-	-
uterus/cervix epithelial carcinomas	-	€ f	-	-	-

m male animals
f female animals

€ increase of tumor rate versus controls
- no effect on tumor incidence

The growth stimulating influence of ethinyl estradiol on the endometrium of mice of the highest dose group (tab. 2) caused a low incidence of neoplastic changes. Also for women an increased risk regarding the development of uterine carcinomas after the menopause is regarded as certain after long-term treatment with estrogens without simultaneous application of gestagens (4,7). The stimulating effect of exogenously applied estrogens on the endometrium of postmenopausal women in whom the endogenous formation of gestagens is negligible may be a biologically plausible mechanism for the increased risk of cancer. Meanwhile a therapy for estrogen replacement is recommended which involves simultaneous administration of gestagens in order to avoid hyperplastic endometrial changes and consequently to reduce the risk of the development of uterine carcinomas (7).

The situation is considerably more complicated if the influence of exogenously applied hormones on the development of carcinomas in other organs and tissues, for instance mammary gland, is to be assessed. In the above mentioned long-term study the rate of mammary tumor in male rats was slightly increased after application of estradiol valerate. The increased mammary tumor rate in rats (according to the literature also observed in mice) after application of estrogens is supposed to be related to the stimulation of prolactin producing cells of the pituitary. Rats and mice show a sensitive positive feedback mechanism between estrogens and prolactin regarding the stimulation of the mammary gland. These regulation mechanisms for mammary gland growth are specific to rodents. As the regulation mechanisms of other animal species and humans are principally different to those of rodents, the tumorigenic effects for this organ in rodents have no predictive value for humans.

A great number of epidemiological studies has been conducted in order to clarify whether there is any relationship between breast cancer in women and the application of estrogens or estrogen/gestagen-combinations. The results of the studies were very contradictory (4,7,8,9) Thus, for example, epidemiological studies suggested a higher risk of breast cancer for 20 to 25 year-old women after intake of oral contraceptives (7,8). However, in an extensive case-control study with women of this age “at risk“ no correlation was found between the duration of hormone intake and the relative risk of breast cancer (10). In the same study even a protective effect was observed for 45 to 54 year-old women correlating with the duration of intake. Although it has to be assumed that endogenous female sex hormones can play a role in breast cancer incidence

in humans, the epidemiological studies have as yet given no convincing indications of an increased risk of breast cancer after intake of exogenous hormones.

Conclusions

The effects of steroidal estrogens on tumor development observed in long-term animal studies have no predictive value, wether quantitative or qualitative, for potential effects resulting from their therapeutical use as contraceptives or for hormone replacement therapy in humans. Nor do the published results of epidemiological studies on the occurrence of breast cancer after hormone intake indicate any unambiguous tumorigenic risk after therapeutical use of steroidal estrogens by humans.

However, it hardly seems possible to extrapolate the conclusions drawn for the toxicologically and pharmacologically well investigated group of steroidal estrogens to other environmental chemicals with estrogenic effects. A separate consideration of “estrogenic“ effectsof a chemical substance and further effects including tumorigenic effects on the mammalian organism seems advisable.

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Endocrinically Active Substances in the Environment: State of the Art

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Summary

Endocrinically active substances are of high ecotoxicological importance. They either act directly upon the endocrine system by binding to an estrogen receptor, or indirectly by acting upon other components of the endocrine system. Substances known to act directly are pesticides, such as methoxychlor, o,p´-DDT and kepone, but also phytoestrogens. Decomposition products of nonionic surfactants alkylphenol polyethoxylates (e.g. 4-nonylphenol) act in a similar way. Organotin compounds, on the contrary, act through an indirect mechanism by inhibiting cytochrome P-450 dependent monooxygenases, e.g. aromatase, which oxidises testosterone to estradiol. The latter is the a reason for the masculinization of some gastropod mollusk, which is a global problem today.

Recent studies in England have revealed that fish living downstream of sewage treatment plants show estrogenic effects. Male fish produce vitellogenin, a yolk protein which is formed under the influence of estradiol and therefore is normally produced by females. Nonylphenol (NP) and other degradation products of alkylphenol polyethoxylates (APE) are apotential cause for these effects. Recent in vivo and in vitro studies point to a slight estrogenic effect. NP is found in the draining channels of Swiss and German sewage treatment plants at usual concentrations of 1-15 µg/l. Other decomposition products (NP1EO, NP2EO, NP1EC) reach concentrations of 1-40 µg/l. Heavily contaminated streams contain an average concentration of 0.3-3 µg/l NP.

To evaluate such concentrations with regard to their estrogenicity in fish populations further experimental studies including field studies are urgently required. Preliminary estimates indicate that concentrations of alkylphenols in drain channels of sewage treatment plants are of ecotoxicological relevance. Furthermore, it has to be assumed that individual APE decomposition products, other chemicals and synthetic estrogens have a cumulative effect. In addition, trace concentrations of synthetic estrogens from contraceptives (17€ -ethinyl estradiol),

which have a considerably higher estrogenic activity, have been determined. So far available data indicate that estrogenic effects cannot be excluded in streams highly polluted with sewage water. As these effects are of considerable ecotoxicological importance, further research in this field is urgently required.

Introduction

Endocrinically active substances are of high ecotoxicological importance because they have adverse affects on reproduction with resulting considerable effects at the population and ecosystem levels. These chemicals should therefore be given great attention. The problem of environmental chemicals which affect the endocrine system has gained great importance in the past years, since unexpected effects have been observed in aquatic systems. At the same time results in toxicology and reproduction biology of human and mammals were published which focused public interest on the problem. Potential causes for increased specific cancerrates and data about decreasing sperm numbers in men during the past 50 years (Carlsen et al. 1992, Sharpe and Skakkebaek 1993) are intensively and controversially discussed today (Save 1994, Stone 1994). The present work will not discuss the latter aspects, but focusses on results in the area of environmental toxicology and ecotoxicology.

The problem of endocrinically active environmental substances has been known for a rather long time (Kupfer and Bulger 1980), and estrogenic substances in plants (phytoestrogens, e.g. in soja) were already found rather long time ago. Here, interest is focussed on the problem of synthetic chemicals. In the fifties, a metabolite of the pesticide methoxychlor was found to cause estrogenic effects in mammals. The toxicity of diethylstilboestrol, DES, a medicament taken by pregnant women in the fifties and sixties, attained a notorious fame - after puberty their daughters developed an increased rate of a rare cancer in the reproduction tract (Stone 1994). A number of further estrogenic effects of this substance in laboratory animals are known. In the sixties, estrogenic effects of DDT were observed in birds (Fry and Toone 1981), and in the past years, severe effects of DDT became apparent in alligators in Florida (Gross and Guillette 1994). In the eighties, the androgenic effect of organotin compounds on gastropods was detected (Gibbs and Bryan 1986, Oehlmann et al. 1992), and in the last years estrogenic effects from drain channels of sewage treatment plants on fish were observed (Jobling and Sumpter 1993, Sumpter and Jobling 1993). Today interest also focusses on the effects of PCB on the hormone system of rats, especially on the suppression of the thyroid gland hormone thyroxin and the resulting increase of the number of spermatozoa. To confirm this, however, further investigations are necessary (Stone 1995). Various negative effects of dioxines on development and reproduction of rats, birds and fish also need to be confirmed.

Here, a review of this field of problems will be given from an ecotoxicological point of view. A very preliminary assessment on the basis of the currently available data and present research needs will be presented. Three groups of environmental chemicals are considered in more

detail: organotin compounds, DDT as well as alkylphenol polyethoxylates (APE) and their degradation products. Considering present knowledge, it can be concluded that there is considerable research need to find answers for this relevant environmental problem.

Mechanisms of Endocrinically Active Substances

Chemicals may either directly act upon the endocrine system by binding to an estrogen receptor, or indirectly via interactions with other components of the endocrine system. Well-known substances with direct effects are pesticides, e.g. o,p´-DDT, metabolites of methoxychlor and kepone, diethylstilboestrol, synthetic estrogens and phytoestrogens. However, degradation products of nonionic surfactants, and alkylphenol (e.g. 4-nonylphenol) act in this way also. Although the chemical structures of these substances are very different, many of these compounds possess a p-substituted phenol group, which is supposed to play a role for the receptor binding. A modification of the number of estrogen receptors can also be considered as a direct effect. Organotin compounds, on the contrary, follow an indirect mechanism, most probably by inhibiting the enzyme aromatase. This enzyme is responsible for the formation of estradiol by oxidation of testosterone.

The physiological effects of the hormone estradiol and of estrogenic substances are manifold. In mammalia the primary target organs are the reproduction tract and the mammary gland. In these organs proliferation and growth of some tissues are stimulated. Estradiol also plays a role within the regulation of glucose and lipid metabolism. In fish, reptiles and amphibians estradiol stimulates the synthesis of the protein vitellogenin. This precursor of the yolk protein of oocytes will be modified by addition of a lipid and phosphate unit before it istransported from the liver to the oocytes of the ovary via the blood. Here it is modified into a structure important for the supply of the embryo with carbon compounds. Vitellogenin is formed exclusively under the influence of estrogen. Therefore, this lipoprotein normally is not expressed in male animals; it may however be formed under the influence of either this hormone or estrogenic chemicals. Accordingly, this mechanism is the basis of most ecotoxicological studies aiming to identify estrogenic substances; here, the vitellogenin synthesis is used as an estrogenic signal.

Important Environmental Chemicals

1. Organotin compounds

Gastropod species occurring in aquatic ecosystems have been damaged world-wide by organotin compounds through the development of male sex organs in female animals with resulting sterility (Gibbs and Bryan 1986). This pseudohermaphroditism is a global problem today and was observed in more than 70 marine species (Oehlmann et al. 1992, Stroben et al.

1992). Organotin originate from antifouling paints (Fent and Hunn 1991) and use as fungicides in different materials (e.g. wood preservatives) reaching surface waters via sewage waters (Fent and Müller 1991). As toxic effects are observed for extremely low concentrations (ppt or ng/l) and since the pollutant sources still exist gastropod populations are exposed to toxic concentrations world-wide. In highly contaminated harbour areas the observed changes in female animals lead to sex dislocation and effects on the reproduction in the snail populations. Further to an increased number of masculinised snails, these populations are characterised by either a decreased number or the absence of young animals (Gibbs and Bryan 1986).

Our investigations show that cytochrome P-450-dependent monooxygenases are inhibited by trialkyltin in fish (Fent and Stegeman 1991, 1993, Fent and Bucheli 1994). As postulated in our studies and indicated by a further study with a marine gastropod (Spooner et al. 1991), inhibition of the aromatase, a cytochrome P-450-dependent monooxygenase, which is responsible for the oxidation of testosterone to estradiol, could be considered as the potential cause for the effect on the reproduction systems in snails. This was been demonstrated recently (see the contribution of J. Oehlmann).

2. DDT

Estrogenic effects have also been found for organochlorine compounds, including the classic example of DDT. Adverse effects of DDT on reproduction of birds, especially the thinning of eggshells and disturbances of the reproduction behaviour have been known since the sixties. The estrogenic effect of DDT is mainly based on the o,p´-DDT isomer that makes out approx. 20 % of technical DDT (Galand et al. 1987). The metabolites o,p´-DDD and DDE as well as hydroxylated metabolites also show estrogenic activities (Kupfer and Bulger 1980). The exposure of gull eggs resulted in a feminisation of all male animals due to the occurrence of ovary tissue in the testes (Fry and Toone 1981). An injection of o,p´-DDT into quail eggs resulted in disturbances of the reproduction behaviour of adult animals and a decrease of the reproduction rate of females (Bryan 1989).

3. Nonionic Surfactants: Alkylphenol Polyethoxylates and Nonylphenol

Recent studies in England have revealed that fish living downstream of sewage treatment plants show estrogenic effects (Purdom et al. 1994). Male fish produce vitellogenin, a yolk protein

which is formed under the influence of estradiol and therefore is typically produced by females. Hermaphrodites have been found as well. Present discussions focus on decomposition products of alkylphenol polyethoxylates (APE). They are considered as a potential cause, since their decomposition products formed in sewage treatment plants (Giger et al. 1984) show slightly estrogenic effects (Soto et al. 1991, Jobling and Sumpter 1993).

Alkylphenol polyethoxylates (APE) are nonionic surfactants, which make out ~ 7 % of the total tensides and ~ 25 % of the production of nonionic tensides in the USA. The annual world-wide production of APE amounts to about 390,000 tons. The most common APE are nonylphenol ethoxylates (NPE), however, octylphenol ethoxylates are used, too. Tensides areamphiphil, i.e. they have a hydrophobic and a hydrophilic part. The activity of tensides results from the alkylphenol group; the p-substituted chain of repeated ethyleneoxide units with different chain lengths forms the hydrophilic part. In sewage treatment plants decomposition products and toxic metabolites such as nonylphenol are formed, which reach sewage sludges and surface waters (Giger et al. 1984, Ahel et al. 1994a). For this reason the use of APE has been prohibited in washing agents in Switzerland since 1986. However, they are still in use in industrial detergents, e.g. in the USA. They are also a component of detergents, dispersing agents and emulsifying agents. NPE and octylphenol ethoxylates are applied as spermicides as well.

Concentrations and Behaviour in the Environment

Alkylphenol polyethoxylates (APE) usually reach surface waters via sewage treatment plants (STP), where they are degraded - but not totally degraded - by microorganisms. In a first rapid step the ethoxylate groups are split off by hydrolysis, and the metabolites nonylphenol (NP), nonylphenol ethoxylate (NP1EO) and nonylphenol diethoxylate (NP2EO) are formed. These metabolites are more toxic than the original substances. Due to the hydrophobic properties of the aromatic group the second step of biodegradation occurs much slower. The interim products can also be biodegraded to alkylphenoxy ethoxylate carboxylic acids (APEC). The second, slower, step of biodegradation, which does not always occur, and the fact that the metabolites are more lipophilic than the parent compounds cause an accumulation of interim products in sludge and sediment. Nonylphenol, e.g. was determined in digested sludge in concentrations between 0,45-2,53 g/kg dry weight (Giger et al. 1984). Approximately 50 % of the APE occurring in the wastewater are estimated to reach the sludge as NP (Brunner et al. 1988). Before prohibition of APE in washing agents NP, NP1EO and NP2EO concentrations between 36-202 µg/l (table 1) were found in drain channels of sewage treatment plants in Switzerland. Today, NP

concentrations are in a range between 1-15 µg/l in Switzerland and Germany; other metabolites (NP1EO, NP2EO, NP1EC) are normally determined to 1-40 µg/l (Ahel et al. 1994a, Ahel et al 1994b, Giger 1990). Concentrations of 15 µg/l were determined in the USA. In highly polluted streams average nonylphenol concentrations are determined to 0,3-3 µg/l (Ahel et al. 1994a, Ahel et al 1994b), but may reach up to > 100µg/l (Blackburn and Waldock 1995). In streams polyphenoxy carboxylic acids products are predominant, whereas in sediment NP was the dominating degradation product. Due to their high octanol/water partition coefficient (log 4,0-4,6) nonylphenol, NP1EO and NP2EO show a tendency towards bioaccumulation in organisms. This was confirmed by residue analyses (table 1). The bioconcentration factor in fish is approx. 300, in one case, however it amounts to 1,300.

Table 1: Environmental concentrations of degradation products of nonionic surfactants

Environmental compartment	Substance	Concentration	Literature
sewage	NP	0,45 - 2,53 (g/kg)* 0,03 (g/kg)*	(Giger et al., 1984) (Giger and Alder, 1995)
STP-drain	NP, NP1EO, NP2EO NP NP1EO, NP2EO	36 - 202 (µg/l) 1 . 10 (µg/l) 1 - 40 (µg/l/	(Stephanou and Giger, 1982) Giger, 1990) (Ahel et al., 1994a)
Streams (Glatt)	NP NP1EO, NP2EO NP1EC, NP2EC	0,3 - 45 (2-3) (µg/l) < 3 - 69 (µg/l) < 2 - 71 (µg/l)	(Ahel et al., 1994b)
Stream sediment	NP	0,5 - 13 (mg/kg)*	(Ahel et al., 1994b)
Fish Algae Duck	NP, NP1EO, NP2EO NP, NP1EO, NP2EO NP, NP1EO, NP2EO	0,03 - 7,0 (mg/kg)* bis 80 (mg/kg)* 0,03 - 2,1 (mg/kg)*	(Ahel et al., 1993)

***** dry weight

Aquatic Toxicity

Amounting to 2-4 g/kg (mouse, rat) the acute mammalian toxicity of APE is low. Dermal toxicity, however, is higher (500 mg/kg), and eye irritation is highest with
5-100 mg/kg. NP can be metabolised to a glucoronide in the body and excreted via the kidney.

Nonionic surfactants are more toxic for aquatic organisms than for mammals. The toxicity of APE increases with decreasing number of ethoxylate units and increasing hydrophobic chain length. Accordingly, the toxicity of the original substances is lower than the toxicity of the metabolites NP, NP1EO and NP2EO, whereas the carboxylic acids are less toxic than the ethoxylates. For

instance the LC50 (48 h) of NP16EO is 110 mg/l for fish (Oryzias latipes) and decreases to 11,2 and 1,4 mg/l for NP9EO and NP, respectively (Yoshima 1986). The LC50 (96 h) for algæ (Skeletonema costatum) is 27 µg/l, and the value for rainbow trouts 480µg/l (Nayler 1992). The no observed effect concentration (NOEC) for reproduction for Daphnia is in the range of 24 µg/l. These data show that the acute toxicity of NP is considerably high.

Estrogenic Effects

Not much knowledge exists about the chronic ecotoxicity of endocrinic chemicals. Therefore, it was a surprising and unexpected finding that decomposition products as NP have estrogenic activity (Soto et al. 1991). Recent in vitro toxicity studies with fish hepatocytes indicate that several decomposition products of APE cause weak estrogenic effects (Jobling and Sumpter 1993, White et al. 1994). Studies based on the vitellogenin synthesis revealed that NP, NP1EO and NP1EC have the same activity (half maximum activity: around 16 µM). In this in vitro test the estrogenic activity, however, is 10^4-10^6 times lower than that of estradiol. The activity of synthetic estrogens (e.g. ethinyl estradiol) was comparable to estradiol (Pelissero et al. 1993). The principal objective of the in vitro tests is the comparative investigation and evaluation of the single substances. The estrogenic potency of single substances, however, may vary according to the used assay. Other in vitro studies give hints on potential differences between fish and mammalia regarding the binding to the estrogen receptor (Thomas and Smith 1993). Kepone- but not DDT isomers - bind to the receptor of fish, which is in contrast to studies with mammals where DDT isomers are bound. However, vitellogenin synthesis in fish hepatocytes is also induced by well-known phytoestrogens.

Studies of the group of Sumpter in England indicate that below drain channels of sewage treatment plants vitellogenin is formed in male fish. Upon 1 to 3 weeks exposure of fish in 15 drain channels of sewage treatment plants a high increase of vitellogenin synthesis was observed (Purdom et al. 1994). It is supposed that the decomposition products of APE, especially NP, are mainly responsible for this effect. The assumption is confirmed indirectly by the results of the in vitro studies with fish hepatocytes. However, it cannot be excluded that synthetic estrogens are also responsible for this effect. On the one hand their concentrations are lower than the usual concentrations of NP, but on the other hand their activity is some orders of magnitude higher. Experimental exposure of fish to NP or metoxychlor (i.p. application) over 7 days induced vitellogenin synthesis in male fish (Nimrod and Benson 1995). The dose required to induce the vitellogenin synthesis was 300 times (approx. 150 mg/kg) higher than the necessary dose of estradiol. The reaction of ethinyl estradiol was more expressed than that of estradiol, however, vitellogenin synthesis was not induced by DDT.

The vitellogenin induction by 17€ -ethinyl estradiol, a synthetic estrogen in contraceptives, was investigated in vivo. At 10 °C rainbow trouts showed a vitellogenin induction at 10 ng/l, at higher temperatures of 16,5 °C induction already occurred at 0,5 ng/l ethinyl estradiol (Purdom et al. 1994). This effect was comparable to the effects observed in fish livingbelow sewage treatment

plants. Further studies indicate that 1 ng/l induces vitellogenin production within 10 d (Sheahan et al. 1994). Unfortunately, no data are available about the concentrations of these synthetic estrogens and their metabolites in wastewater, but it is supposed that their concentration are in this range.

Risk Assessment

Based on the so far available data a preliminary assessment of the situation of wastewater polluted surface waters will be attempted. It has to be pointed out, however, that further research in this field, especially the conduction of experimental in vivo studies, is urgently required to allow for a more reliable assessment of the exposure of fish populations to estrogenic chemicals and their potential effects.

Field investigations indicate that below drain channels of sewage treatment plants estrogenic effects may be induced in fish. It is assumed that the effects observed in England are of general importance. An assessment of the situation in surface waters is more difficult, since respective studies are not available. Therefore an indirect consideration will be attempted. The in vitro studies with fish hepatocytes seem to indicate that the estrogenic activity of synthetic estrogens is some orders of magnitide higher than the activity of decomposition products of APE. On the other hand the estrogenic potency of NP, NP1EO, NP2EO and NP1EC is very similar. Consequently, all degradation products have to be taken into consideration.
It seems advisable to suppose that the above chemicals have additive effects. In the few investigated fish around 0,03-7,0 mg/kg alkylphenols were determined (Table 1). The values (~ 2 µM) determined in fish by Ahel et al. (1993) are about one order of magnitude below the half maximum effect concentration of alkylphenols (~ 16 µM) determined in the in vitro assay by Jobling and Sumpter (1993). Another comparison also points to the fact that the concentrations of NP in fish (Ahel et al. 1993) are approximately one order of magnitude lower than the effect concentrations (vitellogenin production) in an in vivo experiment (Nimrod and Benson 1995). Although no direct conclusions can be drawn from these comparisons they can be referred to giving some important hints.
Taking into account that further to the effects of NP the single decomposition products of APE as well as synthetic estrogens and other compounds most probably have additive effects, estrogenic effects cannot be excluded in surface waters highly polluted with sewage. This assumption seems to be justified since the total concentration of estrogenic chemicals is in a range very close to or within the effects range. As a next step respective experimental and field studies have to be performed to confirm this assumption.

So far, no knowledge exists about environmental concentrations of synthetic estrogens from contraceptives (17€ -ethinyl estradiol). Available data, however, seem to indicate that synthetic estrogens from contraceptives and alkylphenols are of comparable importance in surface waters contaminated with sewage. It should be noted that additional estrogenic compounds may occur

in sewage, e.g. some phthalates and others (Jobbling et al. 1995). This especially applies for dry summer periods with low water levels leading to a high percentage of sewage water referred to the total stream volume. Further ecotoxicological research in this field is urgently required to allow for a profound analysis and assessment of the problem. Respective research work has to include investigations to elucidate the ecotoxicological importance of vitellogenin production in male and juvenile fish and further estrogenic effects on fish populations. Especially the high energy input of the organism for the vitellogenin synthesis has to be taken into consideration. On a long-term basis this may be a relevant physiological stress for the whole organism, since the production of these non-essential proteins may impair the synthesis of essential proteins. Research in this field is urgently required since the considered effects are of high ecotoxicological importance.

Open Questions and Research Gaps

The importance of estrogenic chemicals is high, since they may have serious effects on animal populations. On the other hand it has to be pointed out that knowledge is not sufficient today for a satisfying assessment of this important problem. Answers can only be obtained from comprehensive ecotoxicological research. Some important research gaps are summarized in the following.

1. Effects of endocrinically active chemicals have not yet been systematically investigated in amphibian and reptiles. In this field nearly no knowledge is available.

2. Chemical methods for the detection of traces of synthetic estrogens and their metabolites must be elaborated, since only very few data are available on environmental concentrations, especially regarding concentrations in drain channels of wastewater treatments plants. Furthermore, data material on NP concentrations in drinking water and organisms including humans is insufficient.

3. The ecotoxicological relevance of vitellogenin production in male animals has to be elucidated. Which interrelations exist between the problem of vitellogenin production and further estrogenic and ecotoxicological effects of NP and other chemicals? To answer these questions in vivo experiments using histopathological, biochemical, endocrinological and reproduction biological methods have to be conducted. Furthermore, insufficient information

is available about the bioaccumulation of these chemicals. In a further step the problem should be investigated by more comprehensive field studies.

4. The mechanisms of chronic effects of alkylphenols (modes of action) must be studied in more detail.

5. Finally in vitro assays should be elaborated to identify and estimate the estrogenic activity of existing new chemicals in fish and other organisms.

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Endocrine Effects of Environmental Chemicals on Fish - Current Investigations

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In the last year the feminization of fish exposed to different surface water and waste water qualities in field experiments was subject of an increasing number of press reports. Especially reports from Great Britain mentioned feminization phenomena of caged rainbow trouts exposed to waste water gradients.

The influence of environmental chemicals on sex differentiation in fish and other organisms (amphibia, reptiles, birds) and their potential hazard to wildlife and human has been discussed for a number of years. Systematic investigations treating this problem have not yet been conducted or are still insufficient. Principally, two substance groups have to be considered, namely, environmental chemicals that have endocrine effects, and synthetic hormones and drugs, which reach surface and ground waters via waste water as a result of their prescribed use.

In 1979 investigations were performed by Rathner and Sonneborn (Institut für Wasser-, Boden- und Lufthygiene, WaBoLu) to allow for a statement on potential hazard to human by estrogens in drinking and waste waters. The authors came to the conclusion that there were no hints on any hazard due to the exposure to endocrine chemicals at that time. Their results indicated that the investigated steroids had a lower solubility in waste water than in aqua bidest (tab. 1).

Table 1: Solubility of ovulation inhibiting steroids in bidest. water and waste water (µg/l)

	bidest. water			waste water		
Etynodiol diacetate	263	± 1,75	(N = 3)	232	± 33,0	(N = 6)
Lynestrenole	275	± 4,01	(N = 6)	173	± 41,6	(N = 3)
Chloromadinone acetate	205	± 4,48	(N = 6)	161	± 17,8	(N = 3)
Mestranol	310	± 3,18	(N = 5)	186	± 25,7	(N = 6)
Norethisteronacetate	998	± 2,79	(N = 6)	927	± 44,0	(N = 3)
Norgestrel	1730	± 4,07	(N = 3)	1088	± 179,6	(N = 3)
Megestrolacetate	1710	± 3,37	(N = 3)	780	± 133,7	(N = 3)
Medroxyprogesterone acetate	1910	± 4,27	(N = 3)	1165	± 57,5	(N = 3)
Ethinylestradiol	4745	± 3,62	(N = 6)	4166	± 360,2	(N = 3)

N = number of measurements, table according to Rathner und Sonneborn, 1979

Measurements of degradation rates in an activated sludge model indicated the degradation of gestagens by microbial processes, but the persistence of estrogens like mestranol and ethinylestradiol under test conditions (tab. 2).

Table 2: Stability of synthetic estrogens and gestagens in the water-recycling system of an activated sludge model.

Substances	% of the initial concentration						
	16 h	24 h	36 h	48 h	72 h	96 h	120 h
Norethisterone acetate	28	-	-	-	-	-	-
Chlormadinonacetate	-	40	-	-	-	-	-
Norgestrel	-	30	-	8	-	-	-
Lynestrenol	-	58	-	42	10	-	-
Megestrolacetate	-	-	-	39	19	-	-
Medroxyprogesterone acetate	-	-	-	30	22	8	-
Mestranol	-	100	-	100	100	100	100
Ethinylestradiol	-	100	-	100	100	100	100

according to Rathner und Sonneborn, 1979

Based on the conditions of Berlin West, Rathner and Sonneborn estimated an excretion rate of 7.5 g/d for the synthetic hormone ethinylestradiol, which is a ratio of 1:50 compared to the „natural“ estrogen secretion. Contrary to the theoretically expected values ethinylestradiol was not detected neither in drinking water nor in waste water samples. Investigations conducted in the Netherlands resulted in concentrations of 0.06 ng/l in drinking water and 0.3 ng/l in river water, respectively. These values were also much lower than the estimated values. As the contraceptive effect of ethinylestradiol begins with a daily dose of 10 µg/l, Rathner and Sonneborn excluded a hazard for the population at that time. In 1987 already 2-15 ng/l ethinylestradiol were measured in river waters.

Besides synthetic hormones and drugs the list of environmental chemicals supposed to have an endocrine potential is continuously increasing (tab. 3).

Table 3: Wide-spread chemicals with supposed effects on the reproductive and endocrine system (from: Environmental Health Perspectives, Colborn et al., 1993).

Pesticides			
Herbicides			
	2,4-D	2,4,5-T	
	Alachlor	Amitrole	Atrazine
	Metribuzin	Nitrofen	Trifluralin
Fungicides			
	Benomyl	Tributyl tin	Mancozeb
	Zineb	Maneb	Ziran
	Metiram-complex		
Insecticides			
	-HCH	Carbaryl	Chlordane

Dicofol	Dieldrine	DDT and metabolites
Endosulfan	Heptachlor and H-epoxide	Lindane (-HCH)
Methomyl	Methoxychlor	Mirex
Oxychlordan	Parathion	Synthetic pyrethroids
Toxaphene	Transnonachlor	
Nematocides		
Aldicarb	DBCP	
Industrial Chemicals		
Cadmium	Dioxin (2,3,7,8-TCDD)	Lead
Mercury	PBBs	PCBs
PCP	Penta- to nonylphenols	Phthalate
Styrenes		

In the meantime the list has to be extended by a number of further chemicals and groups of chemicals (nonylphenol, non-ionic surfactants a.s.o). It has to be assumed that endocrine effects and sex differentiation in fish, resp., are induced by additive effects of environmental chemicals even though individual concentrations will hardly be detectable in a particular case. Therefore, potential observable effects on the endocrine system of aquatic organisms principally will not be attributed to single substances except for exceptional cases (e.g. TBT/imposex). Moreover, only little reliable data are available at present on the mode of action of single substances.

The development of the embryo and the reproductive organs are controlled in a complex way and are therefore susceptible at many metabolic steps. As mentioned above, a number of substances are suspected to have endocrine disrupting activity. Especially persistent chemicals with a high potential for bioaccumulation are problematic since they are deposited in the fatty tissue (tab. 3). The compounds may express their toxicity either directly or delayed in time, e.g. if endogenous body fat has to be metabolized due to a food shortage or if fat metabolism will be increased during spawning. In such cases an enhanced release and activation of substances accumulated in the fatty tissue usually occurs. Possible modes of action ofendocrine chemicals are e.g. linkage to specific gen sectors and intracellular receptors. As a result, induction of hormonal activation or blocking of receptors may occur leading to a disturbance of normal embryonic development.
The described impairments of development are well accepted. Moreover, it has to be assumed that at least some of the above mentioned chemicals caninduce alterations in the target organs (e.g. gonads) of mature organisms (vitellogenesis in adult salmonides).

The sex of fishes is usually determined during the egg and larval development. Besides a potential influence of environmental chemicals the following conditions and aspects are of importance for sex determination:

- chemical-physical factors (e.g. temperature, oxygen, pH)
- incubation time (exposure time during egg and larval development)
- species specific characteristics (genetics).

x
Systematic investigations of this subject are not known to us.

In foreign countries aquacultures have been treated with sexual hormones to produce monosex groups of fish. One example is the feeding of fry of *Saroterodon* (“Tilapia”) with methyltestosteron, which is mixed with the diet at concentrations of 3 ng/g dry weight. The

treatment results in nearly 100% males. The example shows that there is principally evidence for effects of endocrinic substances on feminisation of male fish.

Since August 1994 preliminary investigations on effects of endocrinic compounds on sexual differentiation in fish have been performed in our department. Controls of our reference stock (rainbow trout) pointed to a nearly balanced male/female ratio (tab. 4) as it is also generally assumed to be typical for Central European fish species.

The first preliminary experiment should give information on potential effects and on the kind of effects that will result after a 10-weeks exposure of three year-old sexual mature rainbow trouts *(Oncorhynchus mykis*) to municipal waste water (tab. 4). No macroscopic changes were observed in the gonads of both sex. An assessment of the sex ratio was not feasible due to the low number of investigated animals (10/test concentration). The activity of the cytochrom P-450 enzyme system was increased as compared to the controls. Theresults of the residue analyses have not yet been submitted.

The second preliminary experiment using immature two year-old trouts gave comparable results. The number of animals per test concentration was also insufficient to statistically prove the changes in sex ratio (tab. 4). In future investigations the sex ratio will be a useful parameter only in exceptional cases (long-term exposure). A respective long-term study with freshly fertilized fry of trouts (200/concentration) was started in February 1995.

Table 4: Sex ratio of rainbow trouts. a) control, age group II, b) age group II, after exposure to wast e water, c) age group I, after exposure to waste water

a)	stock control	m	:	f	n
	€ 1986 - 1995	1	:	0,91	412
	€ 1994 / 1995	1	:	0,92	304
	€ 1994	1	:	1,02	160
	€ 1995	1	:	0,83	144
b)	1. investigation period (01.08. - 13.10.1994)				
	water qualitiy	m	:	f	n
	stock control	1	:	0,43	30
	33 % waste water (A)	1	:	0,43	10
	33 % waste water (B)	1	:	1,5	10
	16 % waste water (A)	1	:	1,0	10
	16 % waste water (B)	1	:	2,33	10
	control	1	:	4,0	10
	€ waste water exposure	1	:	1,11	40
	€ control	1	:	0,74	40
c)	2. investigation period (31.10.1994 - 09.01.1995)				

stock control	m	:	f	n
66 % waste water	1	:	0,8	27
50 % waste water	1	:	0,65	28
33 % waste water	1	:	0,77	30
16,5 % waste water	1	:	0,72	31
control	1	:	1,14	30

The objective of the ongoing investigations is to experimentally determine the effects of hormones, endocrinic substances und drugs on sex differentiation in fish. For this purpose single substances and combinations will be investigated in laboratory experiments (concentration/effect relationships, fertility tests). In addition, long-term studies using a stream simulation facility will be conducted to allow for a more reliable assessment of possible effects on populations in an outdoor ecosystem.

Another important aspect is the determination of effects parameters. In a first step the suitability of the following approaches will be tested:

- enzymatic (P-450-system)
- genetic (chromosomes)
- histologic (pathology, hormone analysis, vitellogenin concentration of the gonades)
- physiologic (sperm motility and fertility)
- phenotypic/morphologic (sex differentiation).

The planned experiments in the artificial stream system of the UBA (Federal Environmental Protection Agency) will considerably contribute to improve knowledge on the basis of which results obtained with field experiments (fishing, catch data) - and consequently the current situation in our surface waters - can be assessed.
Before attributing potential effects to endocrinic substances, however, thereshould be evidence that any other potential parameters can either be excluded or are known (tab. 5).

Table 5: Factors to be considered for the assessment of field studies

a)	Chem. / phys.factors
b)	Species specific characteristics
	€ gynogenese
	€ hermaphroditism, sex reversal
	€ "natural" sex ratio
	€ length-/age dependency, sex ratio of the population
	€ species dependent, sex specific growth differences
c)	Effects of the used sampling method (draught specific problems)

In the following the items b) and c) will be explained giving respective examples.
Some species of viviparous poeciliidae consist of only females. The insemination by sperms of other species solely stimulates egg development, mixing of the genes, however, does not occur. Similar situations are known for the endemic fish species.
In Germany, populations of the. *Carassius auratus giebelio* (Cyprininae) are frequently found which are almost exclusively composed of females. The females are able to produce offspring without males and to support the population. As this phenomenon was already described in earlier literature, it can be concluded with a high certainty that there is no causal relation with the occurrence of anthropogenic contaminants.

Spawning female fish join with related fish species. After spawning, sperms of foreign species penetrate the eggs, however, fertilization in the original sense does not occur. The penetrated sperm nucleus perishes without preceding fusion, but the egg is stimulated to cell division and egg development. In populations of this species an increased female ratio is not unusual.

Sex reversal is known in some other species and was investigated in detail in studies with *Xiphorus helleri helleri* (poeciliidae). Contrary to other species these fishes do not have sex chromosomes, but the sex determining genes are regularly distributed on all chromosomes. The young, immature animals initially are females and differentiate to males or females in this stage of development. It is also possible that sexually mature females become masculinized. Consequently, the proportion of males and females is not a suitable parameter in such populations.

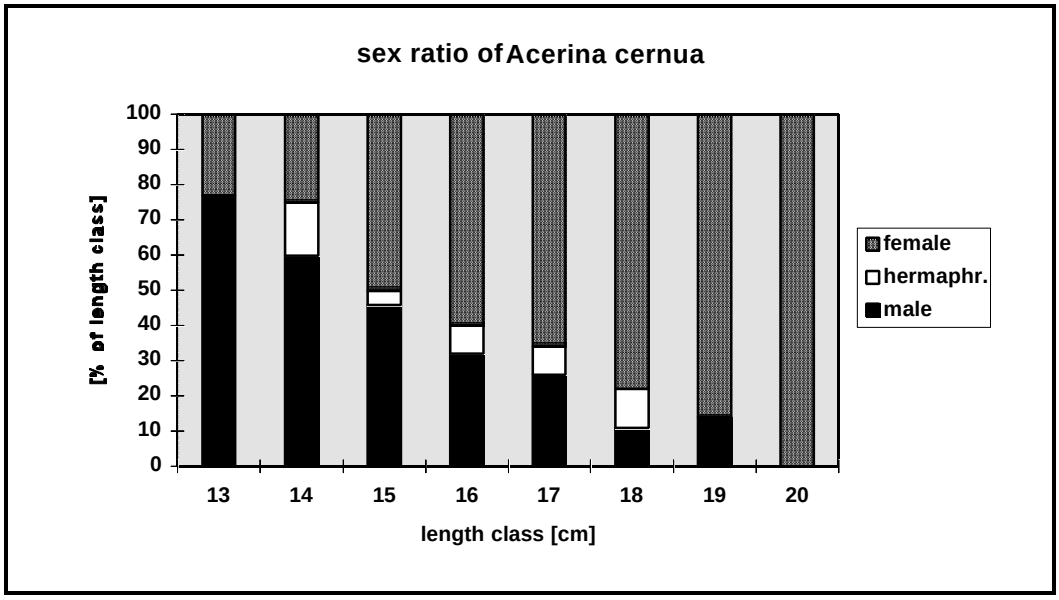
In contrast to the so-called "hermaphroditism in time" a "hermaphroditism in space" (i.e. simultanous development of mature female and male gametes in one fish) also frequently occurs in some fish species. This phenomenon is quite regularly found in some populations of the *Osmerus eperlanus* (osmeridae) and of percidae. More often a juvenile hermaphroditism is observed in some fish species of petronyzonidae, osmeridae, salmonidae and anguillidae. In this case, the gonads of the juvenile stages and larvae, respectively, initially are female and develop to the usually typical sex ratio of 1:1 during maturation. Unambiguous causes for these phenomena have not yet been found.

Cross-breeding experiments with several fish species can also result in offspring predominantly composed of one sex (e.g. *S. mossambica* x *S. marcrochii:* nearly 89% male offspring).

The examples indicate that when assessing the sex ratio in one population genetically related effects and predispositions, respectively, have to be carefully considered.

In some fish species the percent proportions of female and male animals may substantially vary depending on length or age. This is shown in fig. 1 for the *Acerina cernua* of the lower part of the river Elbe (Knowles, 1974).

Figure 1: Percent proportion of males and females in the length groups, *A. cernua*, lower part of river Elbe.


sex ratio of Acerina cernua
[% of length class]
100
90
80
70
60
50
40
30
20
10
0
13
14
15
16
17
18
19
20
length class [cm]
female
hermaphr.
male

In tab. 6 the sex ratios of the *Cottus gobio* (Cottidae) are shown based on comprehensive data (Stahlberg-Meinhardt, 1994).

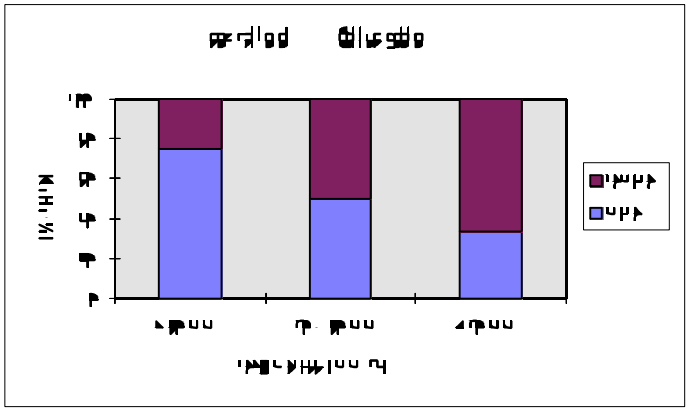
Table 6: Sex ratio of *Cottus gobio*, classified with regard to the year of catch and waters, related to the whole number of all reliably sexed individuals.

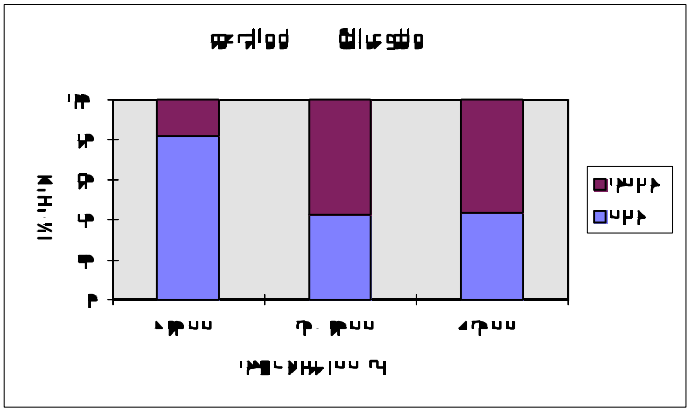
year	water	f		m	n
1990	Sennebach	1.0	:	1.0	810
	Neile	1.0	:	1.3	768
	Steimkerbach	1.0	:	0.8	705
	Neile/Steimkerbach	1.0	:	1.0	1491
1991	Sennebach	1.0	:	1.2	143
	Neile	1.0	:	0.8	356
	Steimkerbach	1.0	:	1.0	264
	Neile/Steimkerbach	1.0	:	0.9	620

according to: Stahlberg-Meinhardt (1994)

In principal sex ratio determinations are representative only if a minimum total number of investigated fishes is given. As for the *A. cernua*, different proportions have been observed for the *Cottus gobio* depending on the inverstigated length groups (fig. 2).

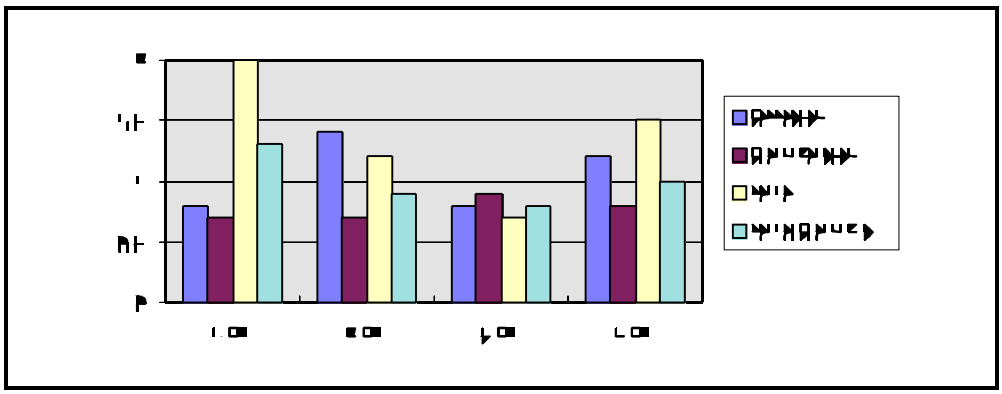
Figure 2: Percent proportion of females and males, classified with regard to size. Top: Sennebach, bottom: Neile/Steimkerbach. The reliably sexed individuals were set 100%; sum: 2301 individuals (Stahlberg-Meinhard, 1994, modified).





Furthermore, fig. 3 shows that depending on the catch period considerably differing values of the sex ratio can be determined in running waters. These may significantly differ from the sex ratio based on the total catch in one year (tab. 6).

Figure 3: Variations of the male proportion, expressed as ratio male/female from different catch periods (FP).



Comparable results were obtained from investigations of lakes (tab. 7).

Table 7: Sex ratio of whitefish (*Coregonus lavaretus*). Determinations were based on samplings using a gill net, 38-44 mm mesh size (Schluchsee, autumn 1983) and on evaluations of non-selected samples.

catch	m	:	f	n
1	1	:	0,72	210
2	1	:	0,66	186
3	1	:	1,13	145
4	1	:	1,6	156
non-selected sample	1	:	0,96	300

Another example for the dependence of the sex ratio of the measured fish length is shown in table 8. Such proportions can be caused by different circumstances. Differences in the maximum final length of males and females of the same fish species - as presented here - or sex specific differences in the growth rate combined with varying strength of the annual population and with

the selection by fishing conditions - e.g. mesh size - can easily result in misinterpretations of the sex ratio of a species, if the data are not carefully evaluated.

Table 8: Sex ratio of flounder (*Platichtys flesus),* from River Elbe estuary (3) end from the southern North Sea, Bay of Helgoland (93).

site	m	:	f	n	length classes [cm]
3	1	:	0,86	54	15,0 - 24,9
3	1	:	1,12	53	15,0 - 24,9
93	1	:	8,0	27	25,0 - 40,0

Summary

- Effects by hormones and other endocrinic substances are principally possible and have already been identified.
- Concentration/effect relationships should be estimated in laboratory studies using single substances and mixtures.
- Appropriate organisms as well as endpoints, and methods, respectively, have to be established and verified (enzyme activities, genetics, histology, physiology, morphology).
- With regard to an assessment of field data it has to be ensured that these are representative (species specific characteristics, distribution in time and space, selectivity of sampling).
- Long-term studies using artificial stream systems under controlled conditions can provide valuable indications for the interpretation of field data.

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Changes in the Sex Ratio of North Sea Dab (*Limanda limanda*) in the Period 1981-1995

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Abstract

In the light of the increasing awareness of the potential impact of anthropogenic endocrine disrupting chemicals in the environment on sexual differentiation, maturation and reproduction of organisms, long-term data from the period 1981-1995 derived from the German North Sea fish disease monitoring programme were statistically analysed for changes in the sex ratio of the common dab (*Limanda limanda*). Combining the data for the whole North Sea, the results of the logistic regression analysis revealed a slightly but significantly decreasing trend in the proportion of female North Sea dab with time. The analysis of regional trends indicate that the decrease in the proportion of females mainly occurred in the central and north-western North Sea, whereas, in contrast, in some parts of the south-eastern North Sea the proportion of females significantly increased.

Indroduction

From a number of studies carried out in the past years there is strong indication that several anthropogenic chemicals and/or their metabolites some of which have been released into the environment since decades (for example DDT) have a potential impact on the endocrine regulation involved in the sexual differentiation, maturation and reproduction of various organisms including humans.

Substances suspected to disrupt the endocrine system are for example synthetic steroids, pesticides, industrial chemicals such as dioxin and PCBs, and alkylphenols (McLachlan *et al*. 1984, Soto *et al*. 1991, Chakravorty *et al*. 1992, Pelissero & Sumpter 1992, Jobling & Sumpter 1993, Colborn *et al*. 1993, Purdom *et al*. 1994, White *et al*. 1994, Kelce *et al*. 1995). Particularly those chemicals acting as oestrogens or antiandrogens by binding to intercellular receptor proteins and evoking hormonal effects are causing great concern in the public since they are suspected to pose a risk to humans (for example DDT and metabolites, Kelce *et al*. 1995).

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In the natural aquatic environment, one of the best documented cases for the effects of endocrine disrupting pollutants is, however, the antioestrogenic effect of tributyltin (TBT) used for antifouling paints on the sexual differentiation of marine gastropods, such as *Nucella lapillus* and *Ocenebra erinacea* (Gibbs *et al*. 1988, 1990) in the North Sea leading to what has been called "imposex" by the development of phenotypic male characteristics in female snails ultimately resulting in infertility of the females and, therefore, possibly in significant effects on the population level.

In studies with caged rainbow trout (*Oncorhynchus mykiss*) experimentally exposed to a waste water gradient in the outlet of a sewage treatment works, an increased synthesis of the yolk protein vitellogenin, which normally is restricted to female fish, occurred in male trout. In addition, single cases of hermaphrodism were recorded. Both effects were attributed to elevated concentrations of oestrogenic alkylphenolic compounds in the effluent, the final products of the biodegradation of non-ionic surfactants during sewage treatment (Jobling & Sumpter 1993; Purdom *et al*. 1994).

Other studies involving fish are for example those by Chakravorty *et al*. (1992) on the effects of pesticides on vitellogenesis, by Pelissero & Sumpter (1992) on steroids and "steroid-like" substances in fish diets, by Moccia *et al*. (1981) on abnormal thyroid function, by Leatherland (1992) on decreased fertility and by Munkittrick *et al*. (1991) and Davis & Bortone (1992) on demasculinisation and feminisation of male fish and defeminisation and masculinisation of female fish, respectively, caused by xenobiotics.

Based on the fact that wild marine fish are exposed to some of the environmental chemicals known or suspected to act oestrogenic and on the possibility that this might lead to disturbances in the sexual differentiation of the fish during early development or even at later stages, the present paper investigates the occurrence of changes in the sex ratio of one of the most

abundant North Sea fish species, the common dab (*Limanda limanda*). Due to its abundance, wide distribution, rather stationary behaviour and the occurrence of several gross external and internal pathological changes, this species is widely used as an indicator organism for monitoring programmes on biological effects of environmental contaminants in the North Sea. In order to detect possible changes in the sex ratio, long-term data from the period 1981-1995 derived from the German North Sea fish disease monitoring programme, a side-product of which are data on the sex ratio of the dab, are analysed for the occurrence of temporal and spatial trends in the proportion of female and male fish.

Material and Methods

Data used for the statistical analysis of changes in the sex ratio of the North Sea dab were obtained from fish disease surveys carried out in the North Sea on board the German RVs "ANTON DOHRN", "WALTHER HERWIG (II)" and "WALTHER HERWIG III" in the years 1981-1995. For most of the years, data from two cruises (January and May/June/July) of each year were available. In total, data from 28 cruises were analysed.
Fish were sampled using standard bottom trawls (GOV and 200/180 ft bottom trawl equipped with cod ends of 20 mm mesh size). Trawling time was one hour and towing speed was 3-4 knots. Besides the examination for externally visible diseases and parasites, the dab were sexed according to external characteristics (size and shape of the gonads which are externally visible on the lower side of the fish) and their total length was recorded to the nearest cm below.

The complete data set used for the statistical analysis of temporal trends regarding changes in the sex ratio of North Sea dab contained information on sex and total length of 269,251 specimens (Table 1). For statistical analysis, the fish were devided into 7 length groups (5 cm width each) representing a size spectrum from 5-39 cm.

The data were analysed by means ANOVA, fitting a general linear model (assuming a binomial error distribution) and applying logit-transformation. In the model, the following effects were considered:

Time **:** Year as a factor with 15 levels was tested as well as a linear and, alternatively, quadratic regression on time.

Size **:** Sex ratio in flatfishes differs systematically with size, leaving more or only females in the upper size classes. This was described as a quadratic regression over the mid-points of the 7 size classes.

Season **:** Differences between winter (January) and summer (May/June/July) samples (possibly due to migration) gives a factor with two levels.

Area **:** In order to identify local patterns, the whole area under investigation (52º - 60º N, 3º W-9º E) was arbitrarily subdivided in rectangles of one degree latitude and two degrees longitude, resulting in an area factor with 34 levels (Fig. 3).

Results and Discussion

As an example for the length/frequency distribution of female and male dab © 10 cm total length, Fig. 1 gives absolut (left column) and relative values (right column) according to cm-groups derived from a cruise in January 1989 for three North Sea areas (area 15: Firth of Forth; area 27: Doggerbank; area 30: German Bight; for location of the areas see Fig. 3). From the figures, it can be seen that the smaller size-groups are dominated by males, whereas the females are more frequent in the larger size-groups. This phenomenon, which is mainly due to the faster growth of females as compared to males, is compensated for in the statistical analysis of time trends, the results of which will be described later.
Again as an example, Fig. 2 illustrates changes in the percent proportion of female dab © 10 cm total length for 6 North Sea areas in the period 1981-1995. The left column shows three areas with a decreasing trend (area 21: northern English coast; area 28: Doggerbank; area 30 German Bight), the right column areas with an increasing trend (area 9: northern Scottish coast; area 19: central-east North Sea off Denmark; area 31: south-western North Sea off England; for location of the areas see Fig.3).

Regarding the statistical analysis of changes in the sex ratio of North Sea dab in the period 1981-1995 based on combined data from all sampling sites, all effects (time, size, season, area) were found to be significant. A temporal trend could be described both as linear and quadratic regression. Both regressions are significant; the former is negative indicating that the proportion of females is decreasing in the period 1981-1995, the latter shows a decrease of the percentage of females after a maximum at approximately 1/3 of the time span. In both cases, the overall reduction in the fraction of females, correcting for the other factors mentioned above, was in the range of 4-5 percentage points.

Looking at trends by single areas (as derived from a model which contains linear time trend and area only as their interaction, all other factors like stated above), some areas mainly located in the central and north-western North Sea were characterized by a significant increase in the proportion of male dab, whilst in others mainly in the more coastal zones of the south-eastern North Sea the proportion of females increased, in contrast (Fig. 3). However, it has to be taken into account that, for some areas, the data set was not complete including only data for some of the years (see Table 1).

Due to the lack of temporal trend data on contaminants in North Sea biota suspected to be endocrine disrupting it is at present not possible to elucidate the role of these compounds in the observed changes in the sex ratio of dab. However, the rare data available (for example North Sea Task Force 1993, de Boer 1995, Dethlefsen & von Westernhagen, in press) for trace organic contaminants in biota suggest that a decrease has taken place in some areas of the North Sea since the end of the seventies, including substances such as DDT and its oestrogenic metabolite *p,p´*- DDE, PCB and -HCH. Based on the (purely hypothetic) assumption that in the period before 1981 the concentrations of these substances might have exceeded no-effect-levels and, therefore, might have caused a shift in the sex ratio towards female dab, the subsequent decline in the proportion of female dab might have been linked with the decreasing xenobiotic concentrations. Thus, this trend could be an indication for an ecological improvement rather than being an indicator for the presence of a persistent adverse biological effect of (possibly antioestrogenic) contaminants notable since 1981. However, the opposite may also be true if one considers the possibility that "new" and endocrine disrupting substances acting antioestrogenic might have been released into the marine ecosystem since the eighties.

Regarding the regional pattern as shown in Fig.3, there is indication that the areas in the central and north-western North Sea which were characterized by a decrease in the proportion of female dab seem to coincide with areas showing elevated concentrations of DDT and its derivates in dab liver tissue in the year 1985 (Büther 1988). The opposite is true for areas with elevated concentrations of PCBs which were mainly located in the southern North Sea, in areas characterized by an increase in the porportion of female dab. However, the data material again is not sufficient to come to any conclusions.

Another anthropogenic factor apart from environmental contaminants with a possible, but yet unassessed impact on the sex ratio of North Sea fish could be the fishing activity. For example, changes in the fishing effort which might have occurred in the years 1981-1995 might have resulted in variations in the size composition of North Sea dab stocks and, therefore, in changes in the sex ratio since an increased removal of large dab would have led to a decrease in the proportion of females versus males, since the large size-groups in dab are dominated by females (see Fig. 1).

In any case, whatever factors might have caused the observed shift in the sex ratio, it has to be taken into account that the decrease in the proportion of female dab in the years 1981-1995 appears to be considerably low (4-5 %). However, the question whether this has to be considered ecologically relevant or not cannot be answered yet.

In order to come to more conclusive results regarding possible changes in the sex ratio of wild North Sea fish and the impact of endocrine disrupting substances, further studies are needed preferably including the measurement of vitellogenesis in male fish and histopathological studies on gonadal development as well as an analysis of long-term changes in the sex ratio of other North Sea fish species than dab in order to test for similar temporal and regional trends. In addition, more chemical background data are urgently needed as well as experimental studies on endocrine disrupting effects of environmental contaminants affecting the reproduction of aquatic organisms.

Table 1: Data used for statistical analysis of trends in the sex ratio of North Sea dab, *Limanda limanda* (season 1: January; season 2: May/June/July; n: number of dab per year and season used for analysis;
areas: areas where dab were sampled, for location of areas see Fig. 3)

year	season	n	areas
1981	1	6.449	24,25,29,30
1982	1	24.666	22,23,24,25,27,28,29,30,31,32,33,34
	2	22.162	19,21,22,24,25,26,27,28,29,30,31,32
1983	1	6.863	16,17,18,19,21,22,24,25,26,27,29,30
	2	18.290	16,17,18,19,21,22,23,24,25,27,29,30,31,32
1984	1	10.205	21,22,24,25,26,27,29,30,31
	2	11.200	17,18,19,21,22,23,24,25,26,27,28,29,30,31,32
1985	1	7.963	19,21,22,24,26,27,28,29,30,31
	2	7.105	19,20,22,24,25,26,27,28,29,30,31
1986	1	13.988	18,19,21,22,23,24,25,26,27,28,29,30,31,32,34
	2	17.770	16,17,18,21,22,23,24,25,26,27,28,29,30,31,34
1987	1	12.743	17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,34
	2	7.714	12,13,14,15,16,17,19,20,21,22,24,25,26,27,28,29,30
1988	1	11.254	21,22,24,25,26,27,28,29,30,31,21
	2	6.588	3,10,15,19,21,25,27,28,30,32,34
1989	1	5.222	1,2,9,10,15,21,25,27,28,30,32,34
	2	6.416	1,3,5,7,8,9,15,16,18,21,22,25,27,28,30,32
1990	1	8.310	1,3,5,6,9,15,21,22,25,27,28,29,30,32,34
	2	6.237	3,8,9,15,17,18,21,22,25,27,28,30,32
1991	1	11.045	9,15,18,21,22,25,27,28,29,30,31,32,34
	2	6.171	9,15,18,21,27,30,31
1992	1	10.072	9,15,17,18,21,22,25,27,28,29,30,31
	2	7.138	3,9,15,18,21,25,27,28,30
1993	1	5.863	3,8,9,15,21,25,27,28,30,31
	2	5.389	3,9,15,18,21,24,25,27,30
1994	1	5.392	9,15,18,24,25,27,28,30,31
	2	2.539	9,10,15,25,27,28,30,32,34
1995	1	4.497	9,11,15,19,21,25,27,30,31,32
	sum:	**269.251**	

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Effects of Endocrine Active Substances on Fish, Exemplified by the Effect of 3,4-Dichloroaniline on the Androgen Metabolism in the Stickleback (*Gasterosteus aculeatus* L.)

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Fishes are exposed to numerous environmental influences. Abiotic factors such as the temperature and the length of day control the cyclic spawning seasons (1,2). Biotic factors such as pheromones, but also visual and tactile stimuli cause, for example, the synchronization of spawning behaviour within one population. Since the industrial age has begun, chemicals in the environment such as detergents and pesticides are an additional anthropogenic factor. All these factors act upon the organism, its development, metabolism, and behaviour.
The factors mentioned above may, for example, influence the function of the organs shown in figure 1 and thus exert an endocrine effect.

Fig 1: General view of endocrine active organs

The olfactory epithelium and the chemosensory organs of the skin and the gills facilitate the perception of pheromonal stimuli (3,4).
Hypothalamus and hypophysis function as master glands.
The brain itself is essential for carrying out the reproductive behaviour. In addition, it contains a steroid aromatase that determines the sex-specific imprinting of an individual by converting testosterone to 17β-estradiol (5,6).
In sticklebacks, the kidney is an accessory sex organ of male fish. In sexual active, i.e. breeding males, it produces a glue, which is required for the nest building (7). The steroidogenic cells are located in the cranial part of the kidney, the head kidney of teleost fishes. These cells correspond to those of the adrenocortex in mammals (8).
The function of the gonads concerning reproduction is obvious.
Liver and kidney play a major part in degradation and excretion of such compounds, as steroids or xenobiotics. The speed of these processes is decisive for the expression of an endocrine activity of endogenous and exogenous substances respectively.
The endocrine system of vertebrates functions like a hierarchically structured communication network. The information is transmitted via blood circulation by chemical messenger substances, the hormones. This humoral coordination controls development, differentiation, metabolic activity and reproduction of the organism. In addition, the reproductive activity of the individuals within one population is synchronized.

Fig. 2: The endocrine system of teleost fishes

The following remarks will mainly refer to the endocrine system of teleost fishes (fig. 2). Exogenous factors such as the temperature, the length of day and, according to recent findings, pheromonal stimuli, are processed by parts of the central nervous system, e.g. the olfactory system. They cause secretion of the releasing hormones such as the gonadotropin releasing hormone (GnRH) in the hypothalamus. Target organ of these substances is the hypophysis. It secretes various gonadotropins, summarised here as gonadotropic hormones (GTH). The effector organ in male teleosts, the testis, consists of two types of cells that respond to different gonadotropins. One type are the steroidogenic or Leydig cells that secrete androgens, above all, testosterone. The androgens control the development and function of secondary sex characters. The other cell type, called Sertoli cells, basically functions as nursing cells for the gametes. Sertoli cells have receptors for both gonadotropin and testosterone as well. These cells secrete a hormone called inhibin which causes negative feedback to the hypophysis. The negative feedback of the Leydig cells is mediated by testosterone and 17β-estradiol, a metabolite produced in the Leydig cells. Testosterone and 17β-estradiol control secretion of the releasing hormone in the hypothalamus. In addition, 17β-estradiol controls the activity of the hypophysis.

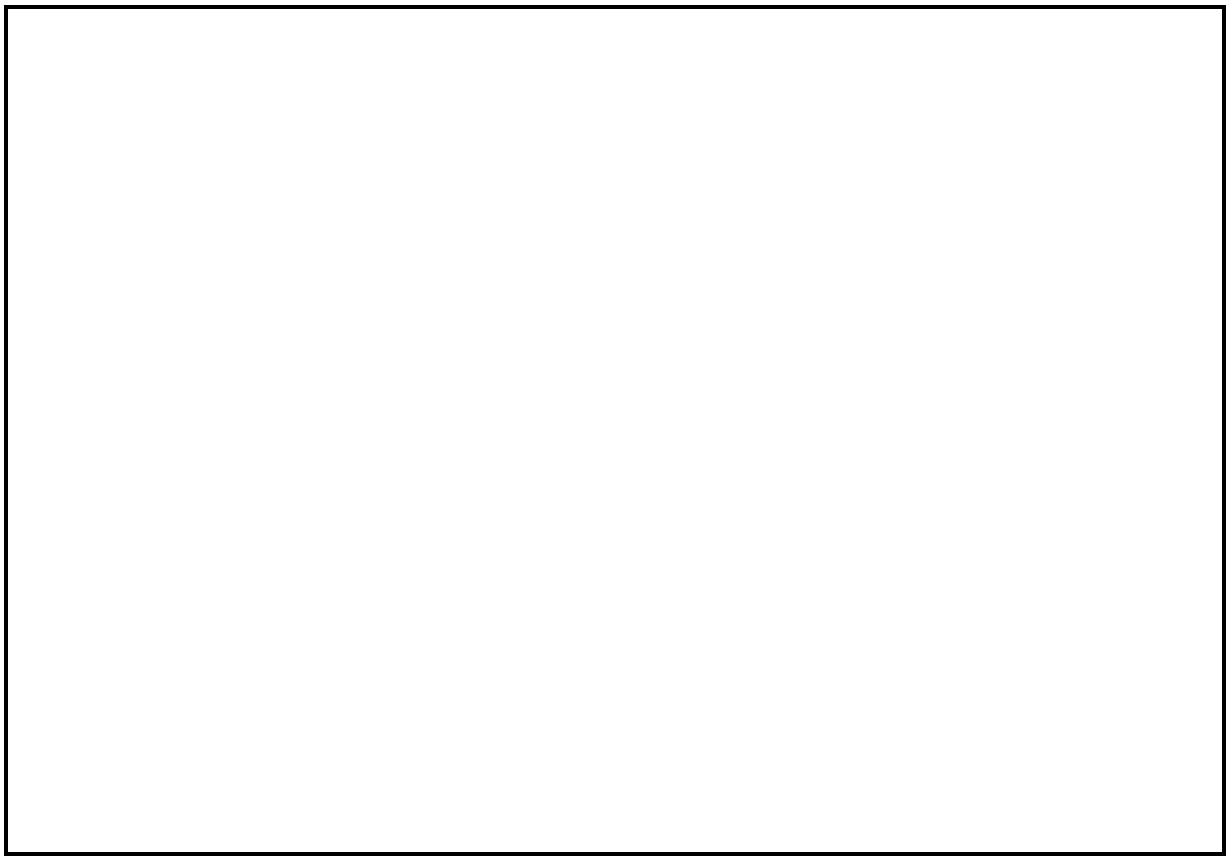
Tab. 1: Endocrine effects of some xenobiotics.

author	substance	organism	action
olfactory epithelium			
Bardach et al., 1965 (9)	linear alkyl benzene sulfonate (LAS)	*Ictalurus natalis*	chemo-reception decreased
Olsen et al., 1985 (10)	"	*Salvelinus alpinus*	chemo-attraction decreased
brain			
Thomas, 1990 (11)	Cd	*Micropogonias undulatus*	GtH increased
"	Pb, Arochlor 1254	"	GtH decreased
gonad			
Sangalang & O'Haloran, 1972 (13)	Cd	*Salvelinus fontinalis*	11KT decreased
Gagnon et al., 1994 (14)	BKME (Bleached-kraft mill effluent)	*Catostomus commersoni*	11KT decreased

Singh & Singh, 1987 (15)	γBHC	*Clarias batrachus*	Testosterone decreased
Freeman et al., 1982 (16)	Arochlor 1254	*Gadus morhua*	Testosterone decreased

In the following some examples will show how exogenous substances may obtain endocrine activity by impairing the function of the organs mentioned above.
It is known for some species that detergents may reduce chemoreception and chemoperception respectively (9,10). For example, chemoattraction of juvenile arctic char (*Salvelinus alpinus*) is disturbed by LAS (linear alkyl benzene sulfonate) at concentrations of 20 µg/L. The question arises whether the perception of reproductive pheromones will be impaired aswell and whether the synchronization of the spawning cycles in a fish population can still be ensured in surface waters contaminated with detergents.
It is known that cadmium and PCBs inhibit secretion of GnRH and GTH (11).

Fig. 3: Accumulation of 3,4-dichloroaniline (3,4-DCA) and the sole metabolite 3,4-dichloroacetanilide (3,4-DCAc) in the brain of the stickleback during 8 hours of exposition to 3,4-DCA



Our toxicological studies on the stickleback have shown that 3,4-dichloroaniline (3,4-DCA) is accumulated in the **brain** in considerable amounts (fig. 3). Dichloroaniline and steroids, at pharmacological concentrations, act as narcotics respectively. The question arises whether the changes in androgen metabolism to be described below are at least partly triggered somewhere on the hypothalamus/hypophysis axis. When testing exogenous substances for environmental impact, comparable distribution patterns or mode of action should give rise to a closer examination of the hypophyseal functions.

Fig. 4: Distribution of free steroids (fr. steroids) and glucuronides after *In vitro* incubation of renal tissue of breeding males using testosterone as precursor. Formation of glucuronide is incresased after 10 days of exposure to 200µg/L 3,4-DCA.

In sticklebacks **the kidney**, as stated above, is an accessory sex organ. Development and function are androgen dependent. The kidney tissue proliferates in breeding males. Non breeding males, similar to females, show a high glucuronyl transferase activity (12). Comparing *in vitro* incubations of renal tissue from non breeding males, breeding males (control) and breeding males, that had been treated with 3,4-DCA, shows that exposure to 3,4-DCA increases the the formation of steroid glucuronides (fig. 4).

In many teleosts steroid glucuronides are not just water-soluble, kidney-passing excretory products of the respective hormones, but they can act as pheromones. In the crucian carp (*Carassius carassius)* these compounds play an important part for the synchronization of the spawning activities (4).

In sticklebacks it was not yet studied whether steroid glucuronides are chemical messenger substances. But our findings should be sufficient to check whether 3,4-DCA is able to impair the chemical communication and the coordination of reproductive processes in fishes.

An overview of the endocrine system of reproduction (fig. 2) shows, that external factors such as light intensity and pheromones may influence, for example, testis functions and in a consequence the development of secondary sex characters via the axis hypothalamus - pituitary - gonads. As a special feature of the endocrine system teleosts have an steroid-11-hydroxylase in the testicular Leydig cells that is otherwise only known from the steroidogenic adrenal tissue of vertebrates (8).

Fig. 5: Some C_{19} steroids with androgen activity found in teleost fishes.

Teleost fishes can therefore synthesize in their testes C_{19} steroids having a functional group at their C_{11} position (fig. 5). 11-ketotestosterone (11KT) shows a greater androgenic potency than testosterone and is considered to be the main androgen in teleosts. 11-hydroxytestosterone (11β-OHT) occurs as an intermediate substance during the synthesis. On the other hand reduction of the 11-ketogroup is a first step towards deactivation of the hormone. Further reductions to androstantriols increase polarity while at the same time decreasing the androgenic activity of the metabolites.

Fig. 6: Distribution of free steroids. *In vitro* incubation of gonadal tissue with breeding males after 10 days of exposition to 200µg, or 400µg 3,4-DCA/L. The values are given in percent of the initial compound.

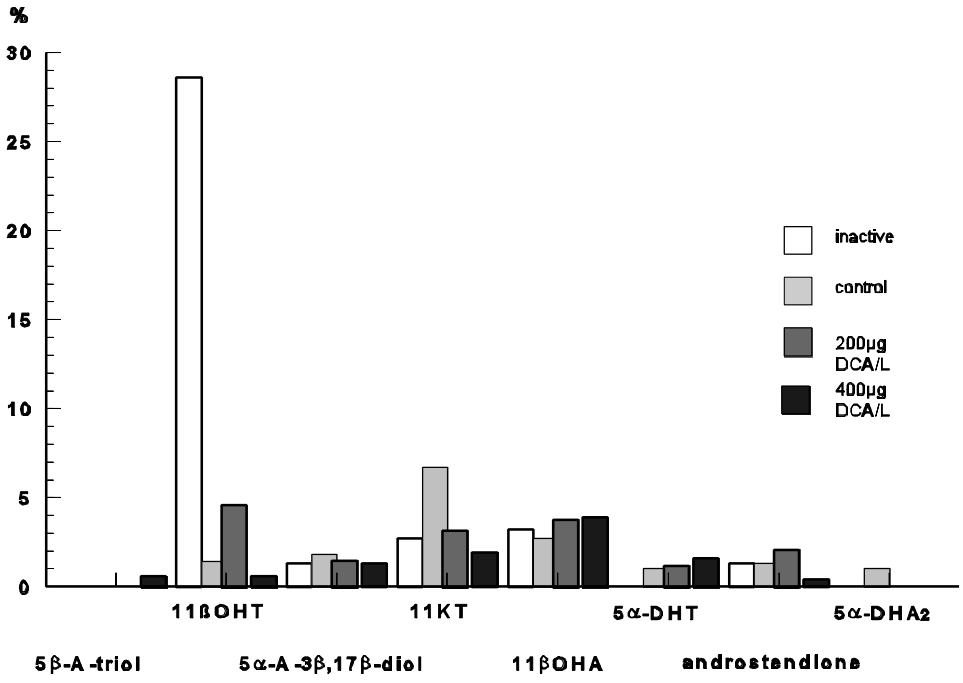


Fig. 7: The total amount of 11-substituted androgens and 11 KT respectively, given in percent of the initial
compound (testosterone).

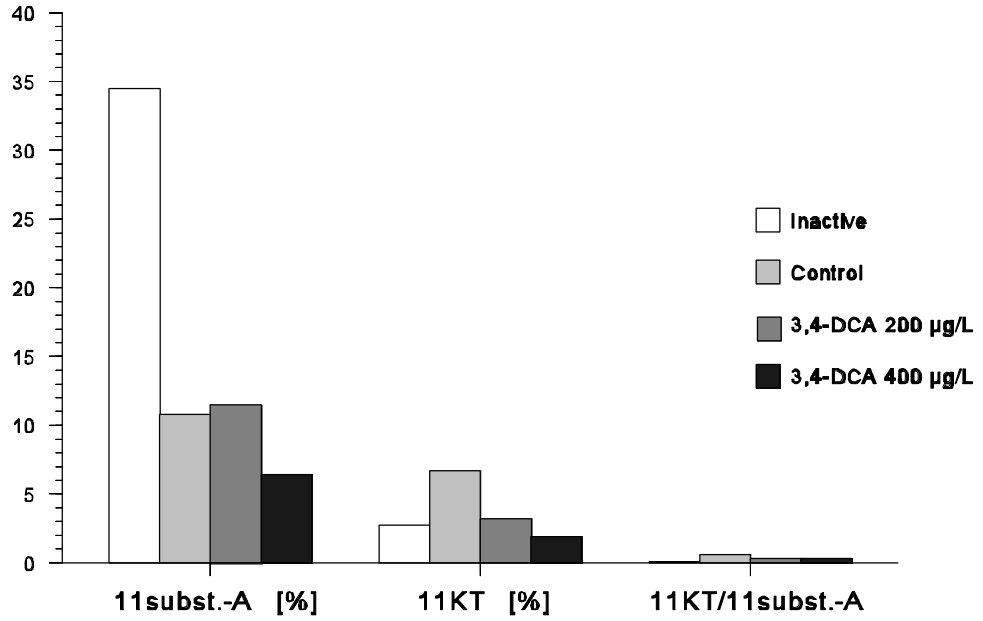


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Fig. 6: Distribution of free steroids. *In vitro* incubation of gonadal tissue with breeding males after 10 days of exposition to 200µg, or 400µg 3,4-DCA/L. The values are given in percent of the initial compound.

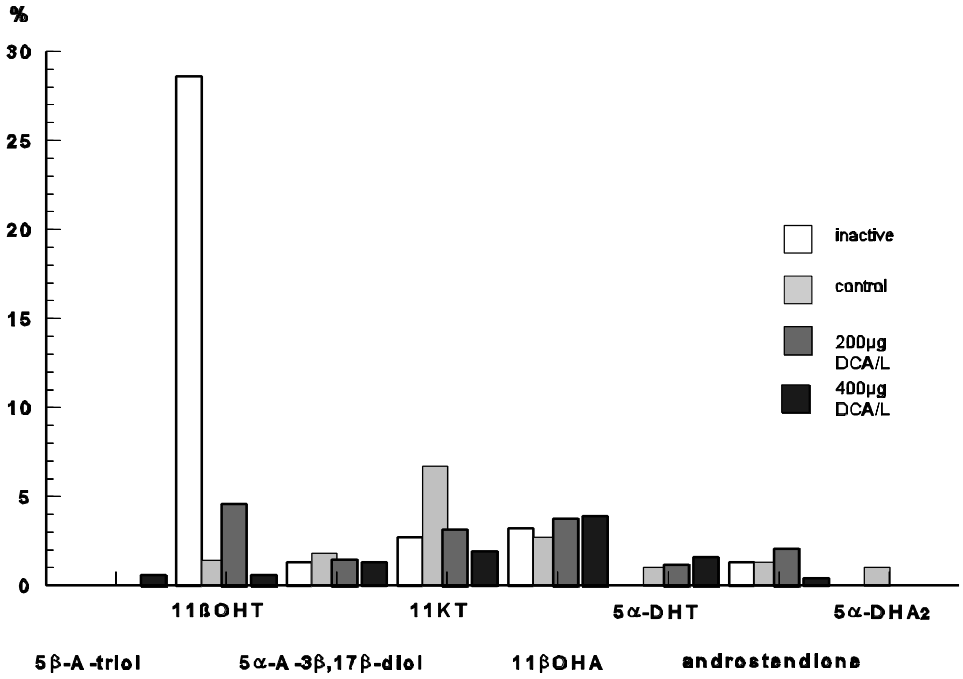
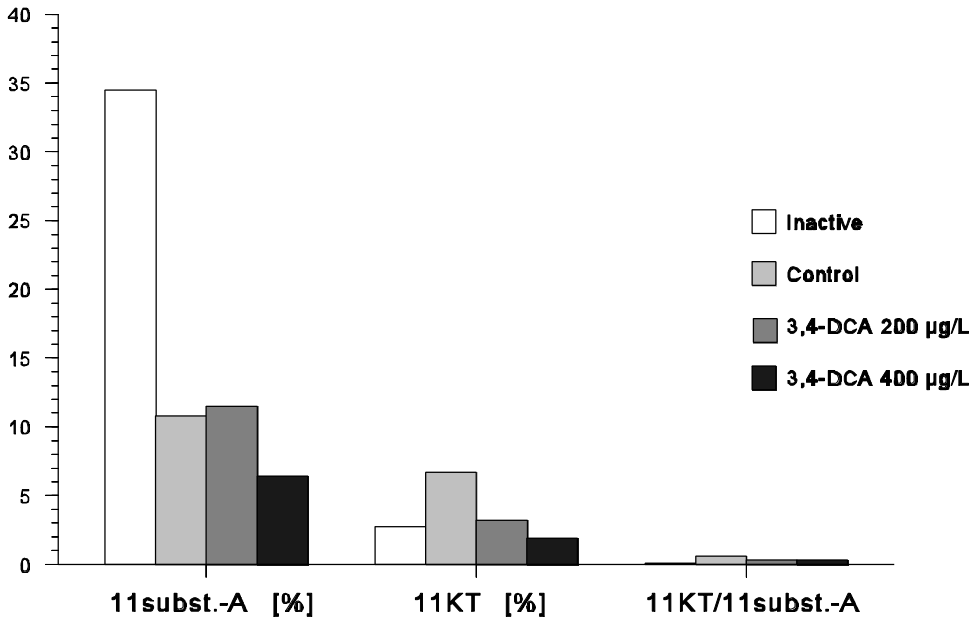


Fig. 7: The total amount of 11-substituted androgens and 11 KT respectively, given in percent of the initial compound (testosterone).



Some disturbances of the gonadal steroid metabolism are known (see Table 1).
The synthesis of 11KT is largely reduced in fish treated with 3,4-DCA (fig. 6 & 7). Initially sexually active animals showed after 10 days of exposition to 3,4-DCA a shift in the range of metabolites, yielding a metabolite pattern that is quite typical of sexually inactive males. Thus fish that were treated with 200 µg of DCA/L form larger amounts of 11-OHT, a metabolite that is increased in inactive males. The changes in androgen metabolism are accompanied by changes in the secondary sex characters. The splendid colour typical of breeding males becomes regressive and courtship behaviour occurs no longer.
The data mentioned above indicate that not only synthetic steroids or their structural analogues can have an endocrine effect but that a great number of xenobiotics is able to impair the endocrine control of fish reproduction. It is highly necessary to take endocrine (toxic) effects into account when testing chemicals for environmental impact.

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Androgenic Effects of Organotin Compounds in Molluscs

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Introduction

Organotin compounds have been produced from the middle of last century and industrially applied since 1950. Tributyltin (TBT) is characterized by a particularly high toxicityamong these compounds. It is considered to be one of the most toxic substances that were ever produced and released into the environment (Müller et al., 1989, Stewart et al., 1992). As itis a highly efficient biocide, it has many applications, for example, in antifouling paints, wood and material preservatives, in textiles, sealing and casting compounds (e.g. polyurethane rigid foams), plastic stabilizers, in paints and adhesives as well as in mineral materials (e.g. insulants) (Bokranz & Plum, 1971, Blunden et al., 1984). Due to this great variety of applications, annual quantities of organotin compounds produced were on a steady rise from just a few tons in 1950 to 35,000 tons in 1985, 5,000 tons of which being TBT (Maguire, 1987).

The greatest portion of TBT produced today is used in antifouling paints. Effects of TBT on aquatic non-target organisms became evident at the beginning of the 1970s. France has enforced restrictions on applying TBT to boats with a length of hull below 25 m since 1982. These restrictions were taken over in 1987 by Ireland and Great Britain, and in 1991 bythe rest of the European Union (Alzieu, 1986, Abel et al., 1987, Huggett et al., 1992). Use of antifouling paints containing organotin compounds in inland waters is prohibited in Germany. Studies carried out after national prohibitions came into force have shown that this did not automatically result in reduced levels of contamination (Kalbfus et al., 1991). That reduction of contamination in coastal waters has been negligible so far, and that we are facing increasing contamination with organotin compounds in limnic environments, is attributed to the expanding applications beyond the antifouling sector as mentioned above. Municipal wastewaters seem to be an important factor in this connection (Fent et al., 1991).

TBT effects in prosobranch snails

Molluscs are particularly sensitive to TBT. The most sensitive TBT bioindicator system is based on the pseudohermaphroditism (Jenner, 1979) and imposex phenomenon (Smith, 1981) in prosobranchs. The females of these gonochoristic species develop additional

Fig. 1: General scheme of imposex development in prosobranchs. Dorsal views with opened mantle cavity. **aK**: mass of abortive capsules; **Gp**: genital papilla; **Kd**: capsular gland; **oB**: open bursa copulatrix; **oK**: open capsular gland; **P**: penis; **PmD**: penis with duct; **Pr**: prostata; **Te**: tentacle; **Vb**:occluded vulva; **Vd**: vas deferens; **Vda**: vas deferens section.

male genital parts, normally a penis and/or vas deferens. Imposex has been described up to now for more than 110 marine species (Fiorni et al., 1991). Development of imposex can be described using the scheme in Fig. 1. This scheme is the basis for the vas deferans sequence (VDS) index that is calculated as the mean value of the imposex stages of a population (Gibbs et al., 1987, Fiorni et al., 1991). Stage 0 is a normally developed female without any male parts. From stage 1 to 4 the size bad extension of penis and for wasdeferens sequence are gradually increasing without influencing fertility. At stage 5, the vagina is supplanted by a prostade gland (stage 5a) or the preformed vaginal opening (vulva) is closed by proliferating vas deferens tissue (stage 5b). Both alternatives suppress the deposition of egg capsules and result in sterility. Abortive egg capsules accumulate in the genital tract (stage 6), the capsule gland is distended until it is torn, which causes death of the specimen (Oehlmann et al., 1991). In other species the ontogenetic closure of the primarily open female genital tract is inhibited (stage 5c), thus suppressing copulation and the formation of intact capsules. This also results in sterility (Gibbs et al., 1990; Oehlmann et al., 1992).

Imposex intensities that are measured, for example, as VDS index rise steeply in the vicinity of TBT emitters in all species examined in detail. A significant correlation ($p << 0.001$) was found between TBT body burden or aquatic TBT concentrations and the VDS index in the dogwhelk, *Nucella lapillus* (Fig. 2).

Fig. 2: Relationship between TBT concentration in ambient water and VDS index in *Nucella lapillus* populations. $y = (5.38 \quad x) \div (0.974+x)$; $n = 131$ populations; $r = 0.678$; $p < 0.0005$.

By the parallel analyses of imposex intensities these species were in different species and the determination of TBT in water and gastropod tissues calibrated as TBT biomonitors. So imposex intensity in a population can give an indication of TBT contamination in its environment (Oehlmann et al., 1993; Bauer et al., 1995; Minchin et al., 1995; current UBA R+D project No 102 40 303).

The described pathologic changes in the prosobranch species examined can be interpreted as virilization phenomena. We first analyzed the steroid content in natural populations to detect the physiological causes for this. We found that advanced stages of imposex due to exposure to TBT in *Nucella lapillus* and other species showed significantly higher androgen levels and testosterone concentrations. This gave rise for the assumption that TBT causes these differences. Changes in the biosynthesis of neurohormones of prosobranchs after exposure to TBT have been known since 1982 (see the overview in Bettin et al., 1995). Female animals produce a penis-inducing factor in their pedal ganglia after exposure to TBT and develop a male mating organ and vas deferens sections.

The effect of three TBT concentrations on imposex development in the dogwhelk, *Nucella lapillus,* was tested in a comprehensive 6-month laboratory experiment. As expected, there was a dose and time-dependent increase in imposex intensity (rise of the VDS index). The VDS index of the control group, however, remained unchanged. Testosterone was added to the laboratory water in another experimental group. This substance proved to be imposex-inducing as well and caused a sharp rise of the VDS index (Fig. 3a).

Fig. 3: *Nucella lapillus:* Development of the VDS index in two test series. (**a**): control; € 500 ng testosterone/l; for comparison with € 5 ng TBT-Sn/l; o 50 ng TBT-Sn/l; 100 ng TBT-Sn/l. (**b**): control group, o 50 ng TBT-Sn/l without and with 1.25 mg cyproterone acetate/l. Sample size : € 30 specimens

Testosterone content in the females was analytically checked at regulat intervals for the three TBT concentrations tested and for the control group. As Fig. 4 shows, there was a clear dose dependent rise in testosterone contents in the groups exposed to TBT. After six months, the females that were exposed to 100 ng TBT-Sn/l contained twice as much testosterone (1,900 pg/g) as the control animals. The differences in the other experiments with exposures to 5 and 50 ng TBT-Sn/l were also statistically significant. This experiment proves that exposure to TBT causes higher testosterone content in the body. Similar findings were reported by Spooner et al. (1991). The experiment cannot reveal, however, whether TBT induces imposex directly or indirectly via raising the androgen level in the system.

Another experiment using cyproterone acetate as an antiandrogen was carried out to solve this question. This substance causes a competitive inhibition of androgen receptors in the tissue by selective binding. It thus prevents coupling of testosterone to the receptors but has no androgenic effect itself.

The animal experiment revealed, as expected, that exposure to TBT alone increases imposex intensity while the test group that was also given cyproterone acetate did not show such increase as compared with the control group (Fig. 3b). This experiment proves that TBT does not cause imposex directly but that development of male genitals in female animals is a secondary effect that is triggered by an increased androgen content.

Fig. 4: *Nucella lapillus*. Mean values and standard deviations of testosterone concentrations in adult females exposed to differing doses of TBT (sample size per group and point in time 6) as a function of the duration of the test. The asterisks indicate significant differences from the control group (Student-*t*-test; $p < 0.01$). Dark column: control; hatched column: 5 ng TBT-Sn/l; dottet column: 50 ng TBT-Sn/l; white column: 100 ng TBT-Sn/l.

The results of these experiments indicate that TBT interferes with steroid biosynthesis at the level of estrogen synthesis. In molluscs, as in all other invertebrates and vertebrates examined, the androgens androstenedione and testosterone are converted into the estrogens estrone and estradiol by a cytochrome P-450 aromatase (Kirchin et al., 1988). This enzyme is part of the multifunctional MFO system that is also responsible for the debutylization of TBT into di-(DBT) and monobutyl tin compounds (MBT) in the organism (Lee, 1988). TBT inhibits the aromatization of androgens into estrogens either directly by deactivation or competitively. This model of TBT activity in prosobranch was confirmed by another series of laboratory tests in which the specific steroidal aromatase inhibitor 1-methyl-1,4-androstadiene-3,17-dion (SH489) showed a similar imposex-inducing capability as TBT (Bettin et al., 1995).

This mechanism of TBT activity explains other effects of this harmful substance, for example, malformations of oyster shells. Delivery of calcium to the shell is also controlled by the MFO system (Lee, 1991). When this system is damaged by TBT, calcium is delivered without control which results in uncontrolled growth of the shells and the "balling" phenomenon.

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In-vivo and In-vitro Methods to Determine the Estrogenic Potency of Environmental Chemicals

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The estrogenic activity of chemicals cannot directly be predicted from their chemical structure. On the one hand, compounds of widely diverse structure are known to exhibit estrogenic activities, as e.g. 17 -estradiol (E_2), zearalenon, diethylstilbestrol (DES), o,p'-DDT and kepone (1). On the other hand, the estrogenic activity of compounds with quite similar structures may considerably differ. This applies for e.g. o,p'-DDT and p,p'-DDT (2) or mirex and kepone (3). The estrogenic potential and the estrogenic potency of a substance can only be determined and characterized by means of biological methods. A substance has an estrogenic potential, when it causes an estrogen-specific effect in a biological system (e.g. in an organism, organ, tissue or cell). The estrogenic potency of a substance is characterized by the dose-dependency of the estrogenic effect. The potency is high if a small dose is sufficient to cause the estrogenic effect; it is low if a high dose is required. The relative estrogenic potency of various chemicals in a test system is expressed as the relation between equally effective doses (see fig. 1) the reference to a standard substance, e.g. 17 -estradiol or diethylstilbestrol, allows to compare obtained to different methods.

At present, no standardized test procedure is available to characterize the estrogenic potency of chemicals. For investigating estrogenic effects and modes of action numerous in-vivo and in-vitro methods are applied. Many of which are principally suitable as test procedures. In the following some methods are presented that have been applied or have recently been used and recommended as test procedures.

Classic in-vivo tests

The classic estrogen bioassays with rats and mice, the Allen-Doisy-test and the uterus-test, have been used since the twenties and thirties to detect the effects of estrogens on the female genital tract (4,5). During the estrous cycle estrogens cause a thickening and cornification of the vaginal epithelium resulting from a stimulation of the cell proliferation rate, as well as a growth of the uterus caused by water retention, hyperplasia and hypertrophy. In juvenile or ovariectomized

females, i.e. animals that are not can able to produce estrogens, such changes can be produced by administration of estrogens. Both tests are based on this principle.

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In the Allen-Doisy-test the cornification of the vaginal epithelium is determined. A substance is considered estrogenic if cornified cells appear in vaginal smears 2-3 days after application of the test substance. The dose dependency of the estrogenic effect can be determined from the frequency of vaginal cornification in groups of animals given different doses.

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- The uterus-test use the weight of the uterus as the endpoint. An increase in uterine weight is a considered to indicate estrogenicity. Fig. 1 shows for illustration the dose-dependent effects of the synthetic estrogen DES, the endogen estrogen estrone, and the phytoestrogen coumestrol in the uterus-test using juvenile female mice.

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Fig. 1: Results of the uterus-test with juvenile female mice. The doses were applied within 4- 6 days with the food. The relative estrogenic potency (RP) was calculated with the dose (ED) causing an increase in uterus weight of up to 25 mg. Data from (6).

Since numerous modifications of the test procedure are possible (e.g. different species, routes of admistration, vehicles, dosage intervals, exposure periods, juvenile or ovariectomized animals, etc.) the results obtained with both test procedures may considerably vary. The results are difficult to interpret unless a simultaneous investigation of a standard, e.g. E_2 is performed.

The natural estrogens were discovered and isolated by means of the Allen-Doisy-test. In the thirties this test was applied to investigate the estrogenicity of a large number of synthetic non-steroid compounds. As a result the synthetic estrogen diethylstilbestrol was discovered, and the estrogenic activity of bisphenols and alkylphenols was recognized (7,8).

The uterus-test has been the most frequently performed estrogen bioassay. It is still in use today. By means of the uterus-test the phyto- and mycoestrogens were discovered and isolated. The test was applied to examine food for estrogenic active residues and is in part still used for

investigating forage in livestock breeding. The estrogenicity of many environmental chemicals, e.g. o,p'-DDT, methoxychlor, kepone and some PCBs, was detected with this assay.

Stimulation of the vitellogenin synthesis in fish and cultured fish liver cells

Vitellogenins are special lipoproteids of oviparous animals. They are produced in the liver of avians, reptils, amphibian and fish under estrogenic control, carried with the blood to the ovaries, taken up there and utilized for the production of yolk proteins. Males and immature females do not synthesize vitellogenin, the synthesis can however be induced by exogen estrogens. Cultured liver cells of e.g. clawed toads and trouts can also be stimulated by estrogens to produce and secrete vitellogenins.

The stimulation of vitellogenin synthesis in juvenile fish was used as endpoint for the determination of the estrogenic potency of ethinyl estradiol in laboratory experiments and for the registration of estrogenic activity in sewage plant effluents in field experiments (9). The results are summarized in fig. 2. Already 0.5 ng/l ethinyl estradiol caused a significant increase in the plasma vitellogenin level, 10 ng/l resulted in plasma vitellogenin concentrations in male trouts that can be found during egg development in mature females. Comparably high vitellogenin levels were also found in juvenile male and female trouts that had been caged in sewage plant effluents over a three weeks period.

Fig. 2: Field experiment: Plasma vitellogenin concentration (mean value) of juvenile female (l,€) and male (n,) trouts, caged in sewage plant effluents or (l,n) in control waters (€ ,)for 3 weeks.
Laboratory experiment: Male trouts were exposed to the indicated ethinyl estradiol concentrations i n aquaria (1600 l, flow through) for 10 days. Data from (9).

According to the authors view the results of the study prove that effective concentrations of estrogenic substances occur in the effluents of sewage plants. Ethinyl estradiol from oral contraceptiva and alkylphenols as degradation products from alkylphenol ethoxylates were assumed to be responsible. However, it was not possible to identify these compounds with common analytical methods.

In connection with these in vivo studies an in vitro assay was developed using primary trout hepatocytes. The endpoint of the test also is the estrogen specific stimulation of vitellogenin synthesis (10,11). Steroid hormones, estrogen metabolites, synthetic estrogens, phytoestrogens and several alkylphenol ethoxylates and their degradation products wereinvestigated using this test system (see compilation in tab.1). Some results are to be mentioned in particular:

- Vitellogenin synthesis was stimulated by nanomolar concentrations of estradiol Considerable differences in the responsivness of the different primary cultures were found (see EC_{50}-values of estradiol, tab.1).

- In contrast to the corticosteroid cortisol progestins (progesterone) and androgens (testosterone) stimulated vitellogenin synthesis; however stimulation occurred at unphysiological high concentrations in the micromolar range (10). This indicates that either androgens and gestagenes have an estrogenic potency as well or that the stimulation of vitellogenin synthesis is not an estrogen specific effect.

- Some of the tested alkylphenol ethoxylates and alkylphenols stimulated vitellogenin synthesis in micromolar concentrations, i.e. with potencies by a factor of 10^4 - 10^{-6} lower than the potency of estradiol.

Tab. 1: Stimulation of the vitellogenin synthesis in cultured trout hepatocytes. EC_{50}: half maximum stimulating concentration. Data in A from (10), in B from (11).

Substance	Relative Potency
A) Estradiol (EC_{50}: ca. 100 nM)	1
Estradiolsulfat	€ 1
Estradiolglucuronid	$2{,}5 \times 10^{-2}$
Ehinyl Estradiol	€ 1
Diethylstilbestrol	7×10^{-2}
Progesterone	ca. 2×10^{-2}
Testosterone	ca. 1×10^{-2}
Phytoestrogenes	$< 1 \times 10^{-3}$

B) Estradiol (EC_{50} = 1,8 nM)	1
4-Butylphenol	$1,6 \times 10^{-4}$
4-Octylphenol	$3,7 \times 10^{-5}$
4-Nonylphenol	$0,9 \times 10^{-5}$
4-Nonylphenoldiethoxylate	$0,6 \times 10^{-5}$
4-Nonylphenoxycarboxylic acid	$0,6 \times 10^{-5}$

In vitro-tests with estrogen sensitive human tumor cell lines

During the last years in vitro estrogen tests using estrogen-responsive cell lines (Ishikawa cells, MCF-cells) derived from human carcinomas have been developed with regard to different objectives.

Ishikawa-cells

Ishikawa cells were derived from a well differentiated human adenocarcinoma of the endometrium. Estrogens cause an pronounced increase in cellular activity of the enzyme alkaline phosphatase (12). Using this endpoint a very feasible microtiter plate assay was developed to determine the estrogenic potency of estrogenic and progestagenic pharmaceuticals (13). Numerous natural and synthetic estrogens and other steroid hormones (androgens, progestagens, corticosteroids) were investigated (13-15). The results obtained so far indicate that the assay is highly reproducible, highly sensitive (EC50-values for E_2 around $10x10^{-12}$ M) and specific (no effects of other steroids at 10^{-6} M). Environmental chemicals have not been tested so far.

MCF-7 cells

Since more than 20 years the estrogen sensitive MCF-7 cell line established from a human mamma carcinom has been intensively used in cancer research and in hormone research to investigate hormone dependent cancerogenesis and the mode of estrogenic and antiestrogenic action, respectively. In the context of these investigations the estrogenicity of several synthetic chemicals was detected. Some of these are phenol red (17), used as pH-indicator in cell cultures, malin of bisphenol-A (18) and nonylphenol (19), both released from polycarbonate and modified polystyrone materials, respectively, was detected.

Today, numerous effects of estrogens on MCF-7 cells are known. Estrogens stimulate cell proliferation and synthesis of estrogen and progesterone receptors, as well as synthesis of several secreted enzymes and growth factors. Using MCF-7 cells two test procedures have been developed for determining estrogenic potency.

Using a test protocol published by Westley and Rochefort (21), Mayr et al. (20) developed an estrogen assay as an alternative to the uterus-test for investigating the estrogenic activity in forage. Synthesis and secretion of a 52-kDa protein (meanwhile identified as procathepsin) is the estrogen specific endpoint of the test. The test is sensitive (EC50-value for E_2: 3×10^{-11} M) and, according to investigations conducted so far, specific (no effects of androgens and progestagens at concentrations of 10^{-6} and higher). However, the laborous procedure for quantifying the 52-kDA-protein (^{35}S-methionine labelling, electrophoresis, autoradiography) is not suited for routine application. So far the assay has been used to determine the estrogenic

potency of natural and synthetic estrogens, myco- and phytoestrogens, as well as for detecting estrogenic activity in forage extracts (20, 21).

The stimulation of the proliferation of MCF-7 cells in the presence of steroid depleted human serum is determined as estrogen specific endpoint in the so-called „E-Screen“ assay established by Soto et al. (22). Using this test system the estrogenic potency of natural and synthetic estrogens, phyto- and mycoestrogens, several pesticides, alkylphenols and naphtols wee investigated (22, 23). Selected results are summarized in tab. 2. Special attention should be paid to the following:

- The proliferation of the MCF-7 cells was stimulated by picomolar concentrations of estradiol. The sensitivity of the test system towards estradiol varied considerably with EC_{50}-values ranging from 1 to 100 x 10^{-12} M (21-23).

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- The ineffectiveness of other steroids was ensured up to concentrations of 10^{-8} M only.

- Some alkylphenols (butyl-, penthyl-, nonylphenol) and pesticides (DDT, kepone, endosulfan, dieldrin, toxaphen) increased the proliferation of the cells at comparable concentrations (max. effect at 1 and 3 x 10^{-5} M, respectively) but with a lower potency (factor of 10^{6}) as compared to estradiol.

Tab. 2: Stimulation of MCF-7 cellproliferation. EC_{100}: lowest maximum stimulating concentration. Data from (22).

Substance	EC_{100}	Relative Potency
Diethylstilbestrol	10 pM	10
Estradiol	30/100 pM	1
Zearalenon	3 nM	1 x 10^{-2}
Coumestrol	3 µM	1 x 10^{-5}
Alkylphenols	10 µM	3 x 10^{-6}
Kepone	30 µM	1 x 10^{-6}
p,p'-DDT	30 µM	1 x 10^{-6}
o,p'-DDT	30 µM	1 x 10^{-6}

Final remarks

For practical reasons, in vitro assays are considered useful screening methods. The presented in vitro-tests are recently developed systems and so far have only been applied by the groups

who had developed them. Their reproducibility in other laboratories still has to be investigated and further optimization is necessary to make them suitable for screening tests. There is also a need to further investigate the specificity of the used endpoints to make sure that exclusively estrogenic effects are detected. When using in vitro data for toxicological assessments is has to be considered that the estrogenic activity and potency of a substance in vivo may depend on pharmacokinetic factors (e.g. resorption/excretion, metabolism, protein binding, accumulation) which are absent or quite different under in vitro conditions.

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Environmental Pollutants Causing Endocrine Disruption- Problems and Possible Solutions

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Pollutants from traffic, agriculture, industry and household are transported over long distances by air and water and contaminate flora and fauna from the Arctic to the Antarctic. Despite a large number of animal experiments, yet little is known concerning the long-term effects of these compounds on wildlife.
In 1990, World Wildlife Fund Canada (WWF) and Canada's Institute for Research on Public Policy (1) published a report on reproductive and endocrine problems among 16 animal species in the Great Lakes region. In all the affected species, including beluga whales, trouts, mink, snapping turtles and sea gulls, elevated concentrations of industrial chemicals and pesticides were detected. Fishes, mammals and reptiles suffered from tumors and malformations as well as disturbances of the hormone and the immune system, metabolism, behaviour and reproduction. Comparing the observed effects with analysed toxicant concentrations and data from animal experiments, it can be concluded that foetal development can be impaired by trace quantities of organochlorine pesticides and PCB's.

The Wingspread statements

In July 1991, Theo Colborn, who synthesised the wildlife research in the Great Lakes region, convened a work session of scientists representing a wide range of research disciplines to share findings about the effects of chemicals on the endocrine system. Of particular concern to the participants in the Wingspread Centre in Racine were man-made chemicals that can mimic sex hormones or interfere with the body's natural hormone system. Even tiny doses of these chemicals can have far-reaching effects on individuals, populations and communities. If present during the early stages of foetal development, especially when the major organs are still being formed and the brain is being programmed to control and respond to hormone signals throughout life, these chemicals may trigger cascades of damaging biochemical changes that affect development of the nervous, immune, endocrine, reproductive and digestive systems. These effects are usually irreversible (2).
In a consensus statement that has had a broad impact on academic research and thinking about chemical policy, it was declared: "We are certain of the following: A large number of man-made

chemicals that have been released into the environment, as well as a few natural ones, have the potential to disrupt the endocrine system of animals, including humans."

A second Wingspread work session entitled "Environmentally Induced Alterations in Development: A Focus on Wildlife" was held in December 1993. The participants declared unanimously: "Declines in a number of species are in progress on the North American continent. Some of these declines are related to exposure to man-made chemicals. Such declines are not solely a US or North America problem but are occurring on a global scale Populations of many long-lived species are declining, some to the verge of extinction, without society's knowledge".

Pesticides and industrial chemicals are suspected to impair immune function, sexual development and reproduction in invertebrates (12), fishes (3) and mammals (9, 27) also in Europe. Marine mammals are especially at risk because they are living at the top of the food chain and accumulate organochlorine chemicals to a very high degree. It was possible to attribute reduced immune function (8) and reproductive failure in harbour seals (*Phoca vitulina*) in the North Sea (10) to the exposure to pollutants, especially to PCB's and other persistent organochlorines. Seals fed on fish from the heavily polluted western part of the Dutch Wadden Sea produced significantly less offspring compared to a control group fed with less polluted fish. PCB's were also reported to produce skeletal malformations and pathological changes in the uteri of seals from the heavily polluted Baltic Sea (15,16,17).

In aquatic and terrestrial ecosystems, the organisms are exposed to complex mixtures of substances, the component parts of which may interact antagonistic, additive or synergistic. Therefore, it is often difficult to identify single compounds responsible for the observed effects. On the other hand, the cause-effect relationship between the impact of tributyltin compounds from antifouling paints and pseudohermaphroditism in marine prosobranch snails, such as the dog-whelk (*Nucella lapillus*) (12) is well established. The imposex phenomenon in the dog-whelk was observed in most of the investigated parts of the North Sea (13). The resulting reduction in fertility is threatening dog-whelk populations in the North Sea. Near the German coast line, only shells of the Wellhornschnecken (*Buccinum undatum*) but no living snails were found (14). Deviations from normal sexual organs of the female Perriwinkle (*Littorina littorea*) were also observed on the east Friesian coast of Germany (6). Live stocks of more than 40 species of neo- and mesogastropods are in danger world-wide.

Many chemicals - few data

The manufacture and use of chemicals has grown rapidly since the turn of the century. The EU Commission lists a total of approximately 100 000 chemicals which were commercially marketed

before the deadline of 18th September, 1981. The available data regarding production volumes, emissions and environmental effects of these chemicals are often insufficient and incomplete.
Chlorine and chlorinated hydrocarbons are among the most common substances in chemical industries. In Germany, three million tonnes of chlorine are used every year for disinfecting drinking water, bleach and production of solvents, PVC, pharmaceuticals, pesticides and special chemicals (18).
For more than 20 years, chlorine based organic substances have been at the heart of controversy because of their persistence, toxicity and tendency to bioaccumulation (21) Although the use of some environmentally hazardous organochlorine compounds such as DDT, PCP, chlordane and PCBs has since been highly restricted or prohibited in most of the industrialised countries (23), legal regulations are lacking for other compounds with comparable properties (e.g. lindane, chlorinated paraffins). The published data on residues in biota as well as the toxicity of chlorinated compounds still in use have raised increasing concern and led to calls for the phase out of the chlorine based chemical production. The Enquete-Kommission "Schutz des Menschen und der Umwelt" of the German parliament at least also agreed in recommending a partial conversion of the chlorine based chemical production (19). The remarkable proportion of organochlorines amongst the substances known to cause endocrine disruption emphasises the call by environmental and consumer organisations for substituting chlorine based products and processes to a large extent (22).
There is an urgent need for action not only with regard to the use of organochlorines but also regarding reduction or elimination of the use of lead, cadmium, mercury, tributyltins, PAH, alkylphenol ethoxylates, phthalates and triazine-herbicides to name just a few examples.

Testing schemes

Underlying and amplifying the concern about emissions and environmental impact of anthropogenic substances is the fact that the currently established methods of toxicity testing do not provide an adequate basis for establishing policies to safeguard against the harmful effects of chemicals to environment and human health. For decades, responsible scientists and government regulators have largely focused their attention on the protection of human health and the control of substances with high acute toxicity. Later on, in the light of bitter practical experiences, the testing schemes were supplemented with teratogenicity, mutagenicity and carcinogenicity tests.
Legislation has been based on monospecies tests with single endpoints, such as mortality or mutagenicity. The predictability of environmental damage by the use of these testing schemes is

limited because in ecosystems, communities of multiple species are exposed to complex mixtures.
Though the number of tests is limited, only incomplete data are available for thousands of "existing" chemicals. In addition, systematic investigations into immunotoxic, neurotoxic, and endocrine effects are missing.
The detection of xenobiotica-induced developmental disruption is problematic. Harmful effects occur before or soon after birth but in many cases do not become fully evident until adulthood. Moreover, the timing of exposure can be crucial: Even a single exposure at a very low dose can have major effects if it occurs at a critical time. This means that short-lived chemicals can also cause impairment of development in humans and wildlife (29).
WWF has warned insistently against ignoring the warning signals noticed so far. The damage to reproductive systems and deterioration of other essential functions could have devastating consequences for life on this planet.

Possible Solutions

In our view the main objectives in this context have to be the avoidance, reduction or elimination, respectively, of industrial chemicals disrupting the endocrine system.
Therefore as a first step, we suggest the following measures, which will provide essential preconditions for the control of these substances:

- Standardisation and international harmonisation of tests for estrogenic and androgenic effects within the framework of OECD and EU. The test requirements for pesticides as well as for existing and new chemicals in the Directives 91/414/EEC and 79/831/EEC should be supplemented with respective assays.
- Systematic screening of pesticides and industrial chemicals to identify endocrine disrupting chemicals and quantify their specific activity.
- Investigations using field and model-ecosystem studies.
- Facility-related collection and publication of emissions, including substances with known hormonal activity (26). For this purpose the pollutant emission register (PER) planned by the EU seems to be principally suitable.
- Introduction of an authorisation scheme for industrial chemicals according to the regulation of pesticides.
- Provision of sufficient financial support for research.

Legislation for chemicals testing must take account of new findings with regard to xenobiotica induced endocrine-disrupting effects and must be adjusted accordingly. New chemicals have to be tested for hormonal activity before being introduced into commerce.

In this respect, the WWF will continue to play its part at national and international levels. WWF's initiative prompted the inclusion of the problem of xenobiotica-induced impairment of the endocrine system on the agenda of the 4th International North Sea Conference (7). In the section "urgent measures to be implemented by the year 2000" the ministers of environment declared:
"To invite OSPAR and the EC as a matter of urgency to launch investigations and/or risk assessments to improve the knowledge of the consequences of substances suspected to have endocrine or hormone-like effects, for example nonylphenol, certain phthalates and certain pesticides, and to adopt necessary measures" (24).
The extrapolation of test results from other species, e.g. on human beingsis clearly limited. The results of the tests performed with 10 to 100 individuals belongingto six or even twelve different species are extrapolated to billions of individuals belonging to 250,000 plant species and one million animal species (5).
In 1992, the contracting parties of the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) have agreed to apply the precautionary principle, by virtue of which preventive measures are to be taken when there are reasonable grounds for concern that substances introduced may bring about hazards to human health or marine ecosystems, even if there is no conclusive evidence of a causal relationship between inputs and effects (25).
Regarding TBT, alkyphenols, alkylphenol ethoxylates, phthalates and PAH there are reasonable grounds justifying action for the reduction or elimination of further releases. Substitution of production processes and products utilising toxic, persistent and bioaccumulating chemicals by clean technology and clean products must be given priority over end-of-pipe technologies. If we do not, we are putting the diversity of life on earth on a fast track to extinction.

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Study on Pharmaceuticals and the Environment

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A: Summary of studies on"Pharmaceuticals and the environment“ described in research literature

A1: Literature research

A2: Discussion with the affected interest groups

A3: Listing of groups of pharmaceuticals that are highly likely to pose a risk for the environment.

B: Work according to a strategy for assessing the ecotoxicologicol risk potential of pharmaceuticals

B1: Presentation of currently accepted strategies for ecotoxicologic risk assessment (e.g. EU Directives on "Risk and Hazard Assessment" for chemicals in the environment, pesticides or biocides)

B2: Check whether such a risk assessment strategy is applicable to pharmaceuticals

C: Check whether data collected under the German Drug Control Act can be used to assess ecotoxicological risk potential

C1: Presentation of parameters suited for exposure assessment

C2: Presentation of parameters suited for action assessment

C3: Case study on ecotoxicological risk and hazard assessment of pharmaceuticals

D: Discussion of the need to amend legislation on pharmaceuticals to cover ecotoxicological aspects

D1: Description of present status of the German Drug Control Act and EU legislation on pharmaceuticals in the environment

D2: Comparison of the European legal framework applicable to pharmaceuticals in the environment with that of other nations (e.g. U.S.A.)

E: Organisation of a workshop on pharmaceuticals in the environment with the participation of the affected interest groups (the industries, doctors/pharmacists, authorities)

A comprehensive survey of this subject can not be realized without the support of colleagues working in the field of "drugs and environment“ since many relevant contributions exist only as "grey literature“. Therefore, we would greatly appreciate to receive informations, important reports, and references to expositions and effects of drugs in the environment. Please send information to Dr. Römbke (adress given in the list of participants), as soon as possible.

Hormonal Active Substances in the Environment: Exposition, Impact and Detection

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State of the art

As a summary of a search in a literature data base it can be stated that results dealing with the endocrine impact of xenobiotics base on epidemiological and out-door data as well as on laboratory test systems. Our present knowledge permits in a few cases in which a high load exists the conclusion that certain chemicals exhibit an endocrine effect on populations.

Effects

Numerous publications in the press and scientific magazines state that there is an impact on fertility in many species and in humans related to the endocrine effect of estrogens and xenobiotics owing a hormonal side effect (Strobel, Dickmann 1993, Stone 1994). The decrease in human sperm count (Sharpe 1993), the change of male and female portions in populations and the change of sexual morphology (Oehlmann 1994, Jobling, Sumpter 1993) as well as the incidence breast cancer (Davis et al. 1993) and other kinds of cancer (Hileman 1994) have been reported.
On the basis of existing information some in vitro tests to assess the estrogenic effect have been developed with yeast cells (Louvion et al. 1993), breast cancer cells (Soto 1993) and liver cell cultures from fish (Jobling, Sumpter 1993).

Causes

Basic correlations between hormonal effect and the existence of chemicals could be proofed in single cases, for example the decrease in fertility of alligators in lake Apopka/Florida related to an accidental release of DDT and the morphological changes of male trout in British rivers related to the release of nonylphenol from sewage plants. In laboratory test systems additional

substances where whown to have an endocrine effect on individuals. This effect is still discussed for many of these chemicals.

The substitution of xenobiotics in recent years has lead to the situation that beneath persistent existing chemicals numerous new products were added to the list of environmental chemicals. During the last decades the number of chemicals in the environment that can be detected has increased due to improved methods. In parallel the detection limits decreased by a factor greater than thousand.
At the same time the funding of environmental monitoring projects has lead to a more complete picture of the load of aquatic and terrestric ecosystems and to a qualitative and quantitative characterisation of possible sources. At the moment there is no urgent need for additional research because the date base is more than sufficient.

Table 1: Pollutants

Elements (toxic)
Chlorinated Hydrocarbons
Hexachlorobenzene
Octachlorostyrene
Pentachlorobenzene
PCB
PCDD/F
PAH (16 EPA)
Selected Pesticides
Aldrin
Cypermethrin
p,p'-DDD
p,p'-DDE
p,p'-DDT
Dichlofluanid
Dieldrin
Furmecyclox
€ -Endosulfan

	€ -Endosulfan
	€ -HCH
	€ -HCH
	€ -HCH
	Heptachlor
	Heptachlorepoxide
	Pentachlorophenol
	cis-Permethrin
	trans-Permethrin

The institute of ecological chemistry has been determining environmental chemicals analytically and from literature including those with hormonal effects (see tab. 1 and Kettrup 1994), thereby granting the experitise for judging the quality of the analytical data. Recently extensive data concerning the situation in the former GDR have been compiled Heinisch, Klein, Kettrup 1991a, Heinisch, Klein, Kettrup 1991b, Heinisch, Klein, Kettrup 1991c, Heinisch et al. 1993). However, estrogens and estrogen like acting substances were covered only scarcely.

Test systems

Appropriate test systems on a biochemical basis or on the basis of individuals are under development. A validation or application of these systems for complex environmental matrices or fractions thereof restricting investigated chemicals to specific groups is still waiting for a solution. In the following three test systems which seem to be interesting for future developments will be introduced.

Detection of estrogen active substances via a genetically engineered yeast

A yeast (*Saccharomyces cerevisiae)* is expressing a chimeric protein containing the hormone binding domain of the human estrogen receptor. By binding estrogens or estrogen-like substances episomal and integrated reporter genes are stimulated via this chimeric transcription activator which contains the hormone binding domain of the estrogen receptor, the DNA binding domain of the yeast GAL4 gene and the activating domain of a viral protein (VP16). Here a

galactose inducible expression vector is used. The induction by steroids can be followed by the colorimetric reaction of nitrophenyl galactoside.
This test system is close to the demand of McLachlan (McLachlan 1993) for a functional toxicology using genetically engineered eucaryotic cells to detect certain functionsand activities of xenobiotics and would thus be one of the first test systems of such a kind.

Detection of estrogenic activity via the growth of a human breast cancer cell line

In 1991 Ana Soto (Soto 1991) realised the proliferation of human breast cancer cell line MCF7 in the absence of estrogen. This proliferation - usually only possiblein the presence of estrogen - was found to be due to the estrogenic effect of nonylphenol an additive in the polymer material used in the laboratory. In the following a test system for substances with estrogenic activity was developed with this cell line.

Detection of estrogenic effects with liver cell cultures from fish

It was demonstrated for fish that male individuals produce vitellogenin if exposed to high doses of estrogen like acting substances. Vitellogenin is a storage protein for egg yolk and under normal conditions only produced by female individuals. An induction of vitellogenin production is also possible with liver cell cultures from fish (Jobling and Sumpter, 1993).

Future Research Aims

Needs

If the assumptions on the impact of endocrine acting substances proof bo be justified an impact on regulatory active populations in the ecosystem earth an human species has to be expected To protect the environment and man there is a growing need to characterise the risk potential of hormonal active chemicals and to develop counter measures if dramatic changes have to be expected. This comprises the risk assessment of existing chemicals by establishing reliable, rapid, low-cost test systems with a maximum interspecies validity of test results. Also, these tests should be regarded in legal regulations for the invention of new chemicals.

Research aims

Davis et al. (1993) and Safe (1994) published lists of chemicals suspected to exert hormonal activities. In addition such lists were discussed during an expert meeting on march 9th 1995 in Berlin at the UBA. At the moment research in performed on that basis because test systems to validate or expand this lists are still under development.

It can be resumed that an inventory of environmentally relevant contaminants with potentially hormonal effect has to be made. For environmental chemicals there already exists extensive information with stress laid on persistent chemicals (see tab. 1).
It has to be clarified for which chemicals there are hints towards hormonal effect, what the fate of these chemicals are in the environment, at which dose levels they are acting and whether existing concentrations are sufficient to exhibit an effect.
For this problem cannot be solved step by step from our point of view, it has to be clarified which test systems are available at the moment to conduct an environmental monitoring based on bioassays. This monitoring has to be performed with complex matrices or appropriate fractions thereof to rapidly detect groups of chemicals with significant effects.
Connected to the support and application of preventive counter-measures to lower the exposition studies of sources (sewage plants, industry, agriculture, geogenic and immission depending back-ground) are only possible with pre-information gained by working.

In the following four questions that will have to be answered in future research are given:

- Which environmentally relevant substances are certainly acting like endocrine substances?

- What are the reactivity and dose levels of these chemicals in the environment?
- What is the load of environmental compartments with respect to hormonally acting chemicals?
- How can safety margins between exposition and effect concentration be established?

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Environmental Chemicals with Hormonal Activity- State of the Discussion and Perspectives.

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This booklet presents the results of the Berlin workshop of March 1995. The aimof this meeting was

- to summarize the internationally available knowledge in the field of endocrine disruptors,
- to reflect this knowledge on the background of the German exposure situation,
- to identify gaps in our knowledge and further research needs,

and not at least to discuss approaches to handle this problem politically.

The interference of environmental chemicals with the endocrine system of humans and animals is probably one of issues discussed most intensive among toxicologists and ecotoxicologists. So the Berlin workshop was only one in a series of international meetings held on this issue:

- The Institute for Environment and Health (IEH) held a „Workshop on Environmental Oestrogens“ on January 30, 1995 at Leicester; UK (1). At this workshop similar topics as in Berlin were covered.

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- From January 23 to 27, 1995 a meeting on male reproductive health and environmental chemicals with estrogenic effects took place in Copenhagen, Denmark. As a result of this meeting a comprehensive documentation on reproductive health and environmental chemicals was compiled (2).

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- The United States Environmental Protection Agency hosted an „Endocrine Disruptor Research Needs Workshop“ from April 10-13, 1995 in Raleigh, NC, USA (3), especially dealing with the relations between endocrine disrupting chemicals and reproductive toxicology, neuro- and behavioural toxicology, immune system and cancer.

The organizers of all these meetings tried to overcome the traditional borders between human- and ecotoxicology and to stimulate discussion among the specialists of different scientific disciplines. These meetings demonstrated how important this co-operation is to develop a new type of a functional toxicology that is able to characterize the risks associated with the use of chemicals.

All meetings also had in common that the scientists assessed this problemvery carefully. There is still a high degree of uncertainty in many cases, both in the findings and in the hypotheses derived thereof. Clear cause- effect relationships can only be drawn in few cases. On the other hand the effects associated with these environmental chemicals such as feminization, decreased fertility, carcinogenicity, changes of the behaviour or interference with the immune system are severe. So it is urgently necessary to check these hypotheses carefully.

Endocrine Effects

The Berlin workshop could by no means cover all aspects of the broad topic „Environmental chemicals with endocrine effects“. The discussion focused almost exclusively on chemicals with effects on the estrogenic system. For environmental estrogens the most interesting hypotheses were presented. On the other hand the discussion showed clearly that the interest of toxicologists and ecotoxicologists should not be restricted on the chemicals interfering with estrogens. The influence of environmental chemicals on the male hormone system or the thyroid systems may be two examples that need further careful evaluation. This workshop could only deal with a small sector of the whole problem of endocrine disruptors.

Substances Causing Effects

The list of substances that proved to interfere with the estrogenic system either in vivo or in vitro test systems comprises besides the natural and synthetic hormones:

a)

- Phytoestrogens,
- Mycoestrogens,
- Pesticides like DDT, methoxychlor, and chlorodecone,
- Chemicals like dioxins, PCBs and alkyl phenols.

The results presented on the workshop, showing the effect of 3,4-dichloro anilinein fish and the recent findings from Ana Soto's laboratory (4) showing the estrogenic activity of some phthalates clearly indicate that the currents lists of endocrine disrupting chemicals are by no means complete.

Exposure

Little is known about the actual concentrations of the substances in the different environmental compartments in Germany. Certainly it must be assumed that natural and synthetic estrogens can be found in waste water. Especially the synthetic estrogens are stable enough not to be degraded in the sewage treatment plant. There may be detectable concentrations in rivers, lakes and maybe even in the drinking water.

British studies made evident that estrogenic activity is present in the run-off of waste water treatment plants. This activity is possibly associated with the presence of alkyl phenol ethoxilates and their degradation products such as nonylphenol.
In Germany the use of these substances is restricted on the basis of a voluntary agreement between the manufacturer's associations and the federal government. Here it is of high priority to examine whether these restrictions are sufficient to protect humans and the environment.

Mode of Action, Biological Tests, and Risk Assessment

Substances acting as hormones can induce irreversible changes during certain stages of the embryonic development in very low doses. These changes can be morphological (e.g. changes in the morphology of the reproductive organs), functional (e.g. lower number of sperms), they can alter the behaviour (e.g. change the mating behaviour) or can have tumorogenic effects. The effects may be delayed and become obvious after a long time in totally different stages of the development. Decreased male fertility caused by a lower number of sperms can only be detected when the organism becomes sexually mature. Chemical exposure and manifestation of the effects may differ by years or even by decades. The test systems that are currently in use for the risk assessment of chemicals have not been developed regarding to these effects. New test guideline drafts of the US EPA and the German Federal Institute for Consumers Health Protection and Veterinary Medicine (BgVV) include endpoints like the reproductive success in two generations and the number of sperms. They may be the first step towards the development of a test strategy. Further test guidelines must be reviewed to include endpoints that may be connected to hormonal activity, screening tests have to be developed and harmonized within the OECD framework and have to be implemented into the chemical test strategies. The four workshops proposed such tests and identified actions that have to be taken.

Up to now only few chemicals have been tested on their hormonal properties and in most cases these tests have been limited to estrogenic action. It is disturbing that the estrogenic effects of alkyl phenols for example were not detected by systematic assessment procedures for existing chemicals, but were found accidentally as the substances leached from plastic vials in Ana Soto's cell culture laboratory.

To date it is not yet feasible to perform an ecotoxicological risk assessment including hormonal effects. The same is true concerning immunotoxic effects and alterations of the behaviour.

For substances that act through the estrogen receptor it could be demonstrated that they act additively (4). Even if a remarkable effect cannot be seen at concentration of a substance relevant in the environment, these combined actions of several chemicals may cause effects. In this case an ecotoxicological assessment of every single chemical would be a professional error. Only the simultaneous assessment of all substances acting in the same manner would lead to an accurate risk assessment.

On the background that

- only little is known on the estrogenic effects of substances,
- almost nothing is known about the influences of environmental chemicals on other hormonal systems (e.g. androgens, thyroid hormones)
- substances with estrogenic properties are likely to act additively,
- only few substances have been tested,
- the development and harmonization of test guidelines is still at its beginning,
- even if guidelines will be available, the testing of all relevant substances will take years or decades and cost millions of dollars

it must be doubted that in this context the traditional risk assessment of single chemicals can provide an adequate protection of humans and the environment. The only promising protective strategy is the consequent minimization of the release of chemicals into the environment - especially of those which are persistent and bioaccumulating. Proposals to replace preventive elements of risk reduction strategies by traditional single substance risk assessments (5) cannot be justified on a scientific basis.

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