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Review of endocrine disrupting natural compounds and endocrine effects of pulp and paper mill and municipal sewage effluents

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Preface

This report has been produced by the Finnish Environmental Research Group in a project conducted by Finnish Environment Institute (FEI) Chemical Division, in partnership with the Finnish Institute for Occupational Health. The project has been funded by the so-called Finnish environment cluster.

The main objectives for the whole project have been as follows:

- to evaluate, at a general level, research on hormonally active environmental chemicals and related phenomena,
- to delineate the field of study and its connections with other related fields of research, and analyse contexts and frameworks of risk assessment and risk management of hormonally active substances,
- to pinpoint gaps in knowledge, with particular emphasis on Nordic conditions, and to identify research needs and related needs for testing and development ,
- to analyse strategic questions for research and testing, and
- to make suggestions for direction of research, particularly from the Finnish point of view.

The objective of the present report is to give a review of the available knowledge of endocrine disrupting natural compounds and endocrine effects of pulp and paper mill and municipal sewage effluents. At the end of this report concluding remarks and research needs are presented. For further clarification of the objective of the present report, it should be stated that this report is a background paper and not a programme for organised research. This review does not give a thorough assessment of risks but is a general analysis attempting to identify some of the relevant principal questions and approaches to answer them.

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Contents

Introduction

In recent years the term ″endocrine disruptors″ has become increasingly common in the vocabulary of professional ecotoxicologists and in the media. The meaning of this term is that a substance(s) after entering the body of an organism interacts with the organism´s hormonal system usually, in some way or the other, by interfering with normal hormonal processes. The occurrence of exogenous chemical substances with endocrinological effects in the body has raised concerns that anthropogenic substances may cause widespread effects in both humans as well as in wildlife.

There are, however, reasons to compare the possible hazard posed by manmade chemicals with substances formed naturally, predominantly in the plant kingdom, in order to get a perspective on endocrinological effects of chemical substances. In fact, hormonal effects of natural substances have been known for centuries in old cultures like those of India and China. For example, extracts from the Neem tree, *Azadirachta indica Juss*., are known to affect more than 200 species of insects for example by growth disruption. Some of the effects are nowadays thought to be caused by inhibition of the release of ecdysone, a steroid insect molting hormone (Tierto Niber 1994).

Although the present review is stressing possible negative impacts of natural substances it is worthwhile pointing out that many natural (as well as man made) compounds have a number of beneficial effects when consumed by humans in the normal diet (Adlercreutz 1995). In the environment, however, wildlife may be facing situations where excessive amounts of endocrinologically active compounds are directly emitted via industrial or community sewage effluents or indirectly via mechanical manipulations of land. This may lead to undesirable negative effects and it is of great importance that our understanding of such events is increased in order to maintain sustainability of natural resources.

2 General background

2.1 Phytoestrogens - a historical review

In the Western culture, the interest towards phytoestrogens was evoked in Australia, when it was found that female sheep grazing on clover became infertile and that the effect-eliciting factor in clover had oestrogen properties (Schinckel 1948). Besides infertility of fertilized female sheep, formation of cysts in the reproductive organs were noted. Together with the fact that unfertilized female sheep and castrated male sheep produced milk it was concluded that impaired fertility was caused by some estrogenic compound in the feed (Shutt 1976). The progress of the disease syndrome, given the name ″clover disease″, was frequently dramatic; at the first widespread epidemic in Western Australia between 1941 and 1944, the fertility of female sheep decreased from about 80 % to 10-30 % (Bennetts et al. 1946). Shortly after attention was directed on the problem it was discovered that similar impaired fertility occurred in both sheep and cattle, both in other parts of Australia, as well as in several other countries (Moule et al. 1963). The width of the problem can be illustrated by the estimates done that during the years to come about one million female sheep of the Australian sheep population failed to reproduce due to the clover disease (Shutt 1976).

From the time Bennetts et al. (1946) showed that a clover species, *Trifolium subterraneum*, caused the impaired fertility in sheep, an extensive research has been directed on the revealing of estrogenic properties of many different kinds of plants used as feed for livestock. It was found that above all various clover species including white clover and red clover, but also other feed plants such as luzern, alfalfa and different grasses contain estrogenic or ″pro-estrogenic″ compounds, e.g. compounds that are themselves inactive, but after consumption may be transformed (microbiologically) to estrogenically active compounds in the intestine (Moule et al. 1963).

In the 1940s and 1950s estrogenic properties of human food stuff such as grain, oats, potato, apple and cherry where revealed by Bradbury & White (1954). These two scientists were the first ones to isolate two potentially estrogenic substances in *T. subterraneum*, e.g. formononetin and genistein. Examples on foodstuffs presently known to contain estrogenically active components are presented in Table 1 (Kaldas & Hughes 1989).

Table 1. Some common plant species containing estrogenically active compounds.

Alfalfa	Yeast	Rape	Garlic
Anise	Coffee	Rice	Apple
Beans (red, green)	Clover	Rye	Peas
Dates	Grain	Salvia	
Grass (several sp.)	Marijuana	Sesame	
Oats	Carrot	Soy beans	
Hop	Parsil	Soy sprouts	
Pomegranate	Potato	Wheat	

It may be noted that more than 300 green plants have been shown to contain phytoestrogens (Mayr et al. 1992). Estrogenic compounds may be found in all parts of plants, in roots, leaves, flowers, seeds and fruits, but the concentrations in different tissues of different species vary considerably (Stob 1983).

Beside a limited number of presently well known phytoestrogens isolated from several of the 300 plant species known to possess estrogenic activity, there are supposedly a large number unidentified chemical structures, as well as transformation products of the substances already identified, with varying estrogenic potential. For example it is nowadays well known that persons using marijuana or coffee are subject to estrogenic compounds. Both coffee and marijuana contain weakly estrogenic compounds. The estrogenic potency of marijuana-extract is expressed as competitive action with 17-β-estradiol at the estrogen receptor in the rat uterus. The effects of coffee-extract is an increase of the relative weight and protein content of the uterus (Kaldas & Hughes 1989). In neither case has anyone successfylly managed to identify the active components.

2.2 Plant derived substances with hormonal effects

A wealth of various chemical structures isolated from plants have consequently been shown to interact with the hormonal system of animals in several different ways. In this instance the probably best surveyed group of compounds are the phytoestrogens, that directly- or after transformation- may produce estrogenic effects. However, except for substances with estrogenic or anti-estrogenic effects, there are many substances occurring in plants that, in some way or the other, affect enzymatic or other functions of the hormonal system. As a consequence, revealing the mode of action of specific biochemical levels of substances is understandably an awkward mission. Thus, both substances usually assigned as ”phytoestrogens” as well as other types of active compounds, be they estrogens, anti-estrogens or having some other hormonal end-point effects, are discussed as an entity in this report.

Phytoestrogens are defined as plant-derived substances that are structurally and/or functionally similar to the sexhormone (steroid) 17-β-estradiol (E2) occurring in animals, or in other ways produce estrogenic effects (Fowler 1983). Following three groups of non-steroid substances are commonly assigned as to belong to the phytoestrogens:

Isoflavonoids, coumestans and lignans. The isoflavonoids (e.g. genistein, daidzein, biochanin A, formmononetin and pratensein) are monocarboxyle-derivatives of flavonoids with a carbon skeleton of 15 atoms, and the coumestans (e.g. coumestrole and 4´-o-methylcoumestrole) also with a 15-C skeleton, are both derivatives of 3-phenylchromane (Figure 1, after Kaldas & Hughes 1989). Isoflavonoids and coumestans are frequently regarded as sub-groups of the main group flavonoids, e.g. they have all a C6-C3-C6 configuration of the carbon skeleton (Rowe 1989).

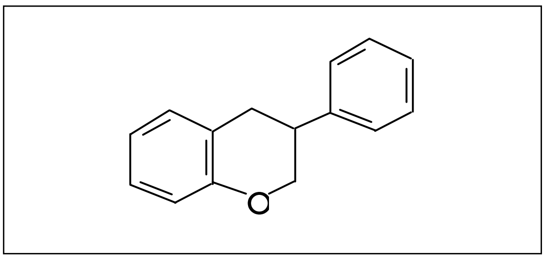

Figure 1. Structural formula of 3-phenylchromane.

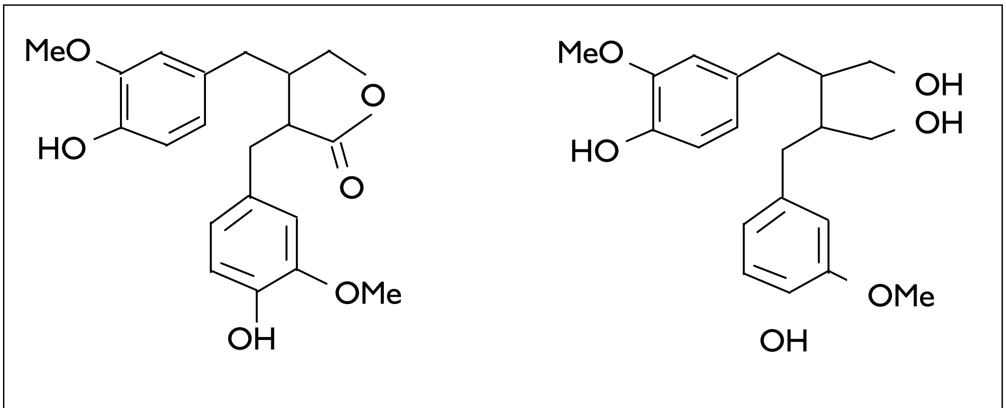

Figure 2. Examples in lignan structures.

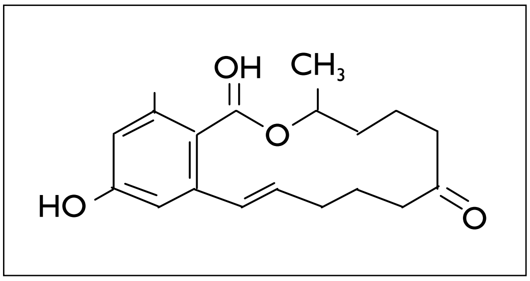

Figure 3. Structural formula of zearalenone.

The lignans have a somewhat different structure, analogous with that of conipherylalcohol, which is thought to be a precursor to the lignans. The lignans have a carbon skeleton based on 18 atoms (Figure 2, after Adlercreutz, 1995).

The so called mycoestrogens (for example zearalenon and zearalenol) are formed by fungi belonging to the *Fusarium* genus and have been identified in grain-based animal feeds (Mayr et al. 1992). These compounds are resocyloxylactones and, like endogenous estrogens, have a carbon skeleton with 17 atoms (Figure 3, after Mäkelä et al. 1994).

Among other interesting chemical structures, despite that they are not regarded as phyto- or mycoestrogens, are the flavonoids, stilbenes (C6-C2-C6), phytosterols and some odd groups like indolecarbinols and resin acids.

2.3 General effects of hormonally active plant compounds

The relative potency of a phytoestrogen to produce an effect depends on which animal species is being exposed, the target organ, the functional state of the tissue as well as exposure route and exposure time. An important aspect, that cannot be stressed enough, is that many estrogenic substances in plants have been subjected to microbial transformation, usually by the intestinal bacterial microflora at least in warm-blooded animals, before the uptake. In several cases metabolites with stronger estrogenic potency than the original substance are formed.

Well known examples on such transformations are the formation of equol in the intestine of animals having consumed isoflavonoid phytoestrogens and the formation of enterodiol and enterolactone in animals having consumed plant lignans (Kaldas & Hughes 1989; Adlercreutz 1995). 50 to 1 000 times increases in urine equol concentrations have been registered in animals having consumed estrogen containing plants, whereas only traces of the isoflavonoids are found in the plants. This is of great importance since many mammals are able to absorb specifically equol, which thereby can enter the enterohepatic circulation and exert its estrogenic effect.

However, it has also been observed that all humans do not have the ability of transforming isoflavonoids to equol, which may be a consequence of that such individuals for different reasons lack isoflavonoid transforming intestinal bacteria (Kaldas & Hughes 1989).

Phytoestrogens (or their transformation products) principally exert their impact by binding to the target organism´s estrogen receptors. Evidence of this has been gained by a clear dose-response relationship between the given dose of phytoestrogens and the fraction unbound radiolabelled E2 in experimental organisms (Verdeal et al. 1980). This means that the structural demands are met for the binding by phytoestrogens to the estrogen receptor.

Due to the considerable quantities of phytoestrogens that normally are consumed by many mammals including man, it is probable that functionally a considerable fraction of the estrogen receptors are occupied by phytoestrogens. Hence, the lower affinity of phytoestrogens to the organism´s receptors may be compensated by their frequently high concentrations.

The effects of phytoestrogens may be either estrogenic or anti-estrogenic. In an individual with low endogenous estrogenic activity the relatively weak xenoestrogens may induce estrogenic effects by their binding to the receptors. On the other hand, in an individual with normal endogenous estrogen level, large amounts of xenoestrogens may instead lower the effective estrogen-actvity by competing with E2 for its normal binding to the receptors (Kaldas & Hughes 1989). In addition to direct interaction with the estrogen receptors in organisms, the phytoestrogens seem to stimulate synthesis of sex hormone binding globulin (SHBG) in the liver and through this action strongly interact with the activity of sex hormones (Adlercreutz et al. 1991). This hypothesis is supported by the observation that vegetarians usually have higher levels of SHBG than non-vegetarians.

The general effect picture at exposure of mammals to phytoestrogen seems to be their impact on practically every aspect of the reproductional process of mammals through effects on the morphology and physiology of the reproductory organs and through changes in reproductional behaviour. Such changes may be reversible or irreversible depending on the duration of exposure and dose (Kaldas & Hughes 1989). It is also supposed that phytoestrogen have a protective effect against sex hormone related cancers such as breast or prostate cancer although the exact mechanisms are so far not fully understood (Adlercreutz et al. 1991).

In addition to effects on specifically reproduction and sex hormone related diseases, it has been observed that phytoestrogens also affect the synthesis of a number of other proteins than SHBG, hepatic enzyme activity and the concentrations of plasma inorganic constituents. They have also been shown to act both as viral and tumour growth inhibitors as well as general growth inhibitors (Setchell & Adlercreutz 1988).

3 Short description of the endocrine system in fish

Since main emphasis in this review is laid upon effects of endocrinologically active compounds on aquatic life and the main component studied in this respect is fish, a description of the endocrine system of fish is given below. In order point out some obstacles regarding interpretation and evaluation of possible endocrine effects in fish, some comparative and general aspects are also discussed.

The endocrine system is primarily involved with chemical signals communication, and it regulates and co-ordinates a number of physiological processes such as reproduction, growth, body homeostasis and energy allocation (Table 2 , Van Der Kraak et al. 1998a). It falls beyond the scope of this review to treat every class of hormones in detail. A more detailed description of the structure and function of different categories of hormones may be found in Van Der Kraak et al. (1998a).

Table 2. Hormones and target tissues/responses involved in regulating major physiological processes (from Van Der Kraak et al. 1998a).

Process	Hormones	Target tissues/Responses
Reproduction	Androgens, estrogens, progestins pituitary hormones (LH, FSH, hCG, prolactin), prostaglandins, growth factors	1. Growth and maintenance of 2. Gamete production, pregnancy, lactation 3. Establishment of secondary sexual characteristics and sexual behaviour
Growth and development	Growth hormone, thyroid hormone, insulin, glucocorticoid, androgens, estrogens, growth	Widespread actions on growth and development.
Maintenance of Internal	Vasopressin, aldosterone, parathyroid hormone, vitamin D3, prostaglandins	1. Control of fluid volume and blood pressure 2. Control of electrolyte balance 3. Control of bone, muscle and fat
Regulation of energy	Insulin, glucagon, thyroid hormones, catecholamines	Regulation of anabolism/catabolism

Hormones are considered highly conserved within the Phylum Vertebrata (Van Der Kraak et al. 1998a). This does not necessarily mean that all types of hormones would be conservative between all categories of animals, which should be kept in mind when extrapolations between different groups of animals are to be done (Campbell & Hutchinson 1998).

In vertebrates the amines and thyroid hormones are identical across vertebrates, as are the majority of steroid hormones. Steroid hormones are synthesized by common pathways, and when differences are occurring, they are frequently relatable to changes in the metabolism of a common precursor (Van Der Kraak et al. 1998a). For example, testosterone is the precursor leading to 11β-hydroxy- or 11-keto-androgens, which are the dominant male androgens in fish, whereas the most active metabolite in mammals usually is 5α-dihydrotestosterone.

3.1 Basic physiological principles on the regulation of reproduction in fish

Fish reproduction and its regulation is here divided into two categories, i) maturation and reproduction, and ii) determination of sex. Fish being poikilothermal organisms, e.g. organisms with a body temperature following that of the external medium, are adapted to changing environmental conditions. Hence, the external environmental conditions affect both sex determination as well as maturation and reproduction. The precise endocrine mechanisms that influence the reproductive success of a species are diverse and are affected by a range of physicochemical and biological factors. The fundamental importance of individual reproduction in assimilating the effects of various environmental factors as exemplified by Campbell and Hutchinson (1998) are presented in Figure 4.

3.1.1 Maturation and reproduction

The sexual maturation process in fish encompasses development of gonads, gametes and secondary sexual characteristics. The sexual maturation is connected with growth and is initiated already at the first intake of food. The simple hypothesis that fish strive at maturation as early as possible, but that physiological (and physical) factors are slowing down the process seems to hold true for the majority of fish species. The maturation process is growth-dependent and completion of the process depends on environmental factors such as temperature and day length. The process may be reversed every year if the environmental conditions are unfavourable. Whether the maturation process is reversed or not, depends on the individual´s available energy resources during certain critical periods of the year.

This mechanism, which is based on that enough parental energy must be available in order to support the progeny, influence both on the primary reproductional processes as well as on the vitality and survival of the larvae during the first winter period. In case of lacking energy reserves, reproduction will not take place. For northern freshwater and marine species, the annual ″window″ for successful reproduction is relatively small (Wootton 1984). Seasonally, the position of the window is presumably connected with the amount of available food for the newly hatched generation.

The gonadal maturation is stimulated and regulated by gonadotropins released by the hypophysis, which in turn is regulated by gonadotropin releasing hormones (GnRH) by hypothalamus. The production of GnRH is dependent on stimulatory action of estrogens on hypothalamus. Juvenile fish produce estrogens by aromatization of kidney androgens. In higher vertebrates such aromatase enzymes occur in lipoid tissue. Their occurrence in fish lipids has not yet been demonstrated, although such enzymes occur in fish brain tissue. It seems plausible that gonadal growth during maturation is controlled in the same way as in higher vertebrates (Thorpe 1994). Sexual maturation in fish may thus be controlled by the individual lipid dynamics (energy balance) during the previous winter and spring.

Gametes are produced in gonads, sperm in the testes and egg cells in the ovaries, and the process is called gametogenesis or gamete formation. Related processes in the cell nucleus are called meiosis. During the normal cell nuclear divisions (mitosis) the daughter cells obtain precisely the same chromosome number as the mother cells. During meiosis or the reductional division that takes place before the gamete formation, the number of chromosomes are halved, however.

In the male sex a great number of cells in the testis (primary spermatocytes or spermatogonia) are undergoing meiosis and each one formes **4 cells** (spermatids) with a halved number of chromosomes. These cells are thereupon, without further divisions, transformed to sperm cells or spermatozoa.

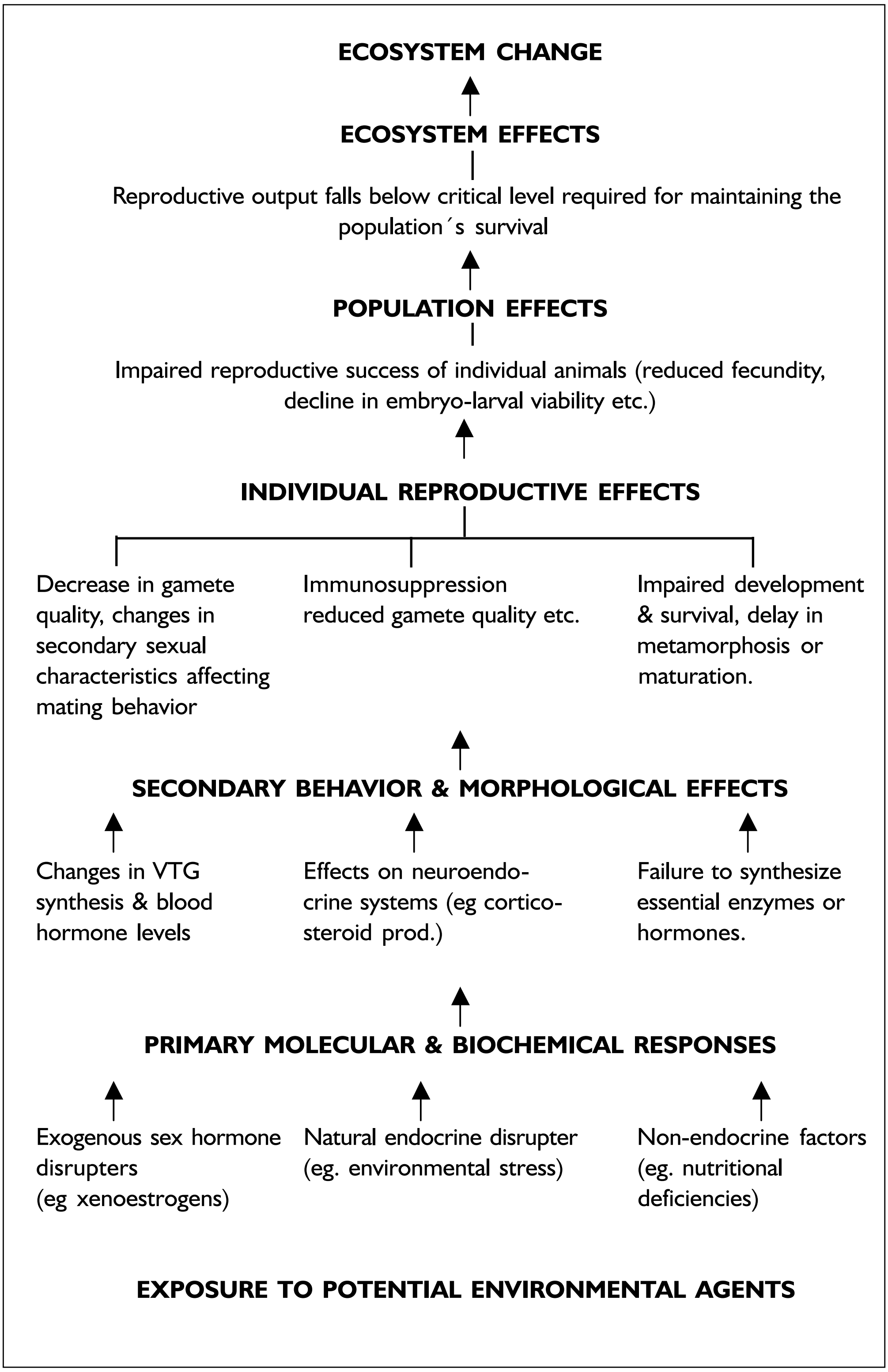

Figure 4. Mechanisms by which endocrine disrupters and other environmental agents affect the reproductive health and survival of aquatic wildlife populations.

In females, meiosis takes place in the ovary, but each mother cell (primary oocyte or oogonium) gives rise to only **one** functional product with a halved chromosome number, whereas the other three cells degenerate. The remaining cell develops to an **egg.** Characteristical for an egg, apart from the half number of chromosomes, is the large amount of stored energy. In other vertebrates than mammals (for example fish) the oocyte grows during the first meiotic phase and this stage is called vitellogenesis.

During vitellogenesis a transformation and storage of a yolk sac protein produced by the liver is taking place in the egg. 17β-estradiol, produced in the gonads under influence of gonadotropin, is released to circulation and stimulates hepatic synthesis and release of vitellogenin. Injection of pituitary extract or gonadotropin induce synthesis of 17β-estradiol and consecutive vitellogenesis. Exogenous estrogens may induce vitellogenesis and increase plasma vitellogenin concentrations. Several hormones such as gonadotropins, thyroxin, insulin and growth hormone have been shown to regulate oocyte growth, although the exact role of none of the hormones mentioned has been specifically determined. Oocyte growth is followed by a process called oocyte maturation taking place before ovulation and is necessary for a successful reproduction.

The spermatogenesis in fish takes place in testicular cysts formed by Sertoli cells. In several fish species the plasma testosterone and 11-ketotestosterone are high during the later part of spermatogenesis. Spermatogenesis is initiated by gonadotropin released by the hypophysis. The effect of the gonadotropin is mediated by an activation of the somatic cells in the testis, that produce 11-ketostestosterone in cells called Leydig cells. 11-ketotestosterone is also causing a prominent activation of the Sertoli cells, but not of the Leydig cells, indicating that this steroid is influencing on spermatogenesis by an activation of the Sertoli cells. The next phase in the male maturation process is spermiation, by which is meant the release of the male sex cells to the sperm duct and further to the surrounding water.

3.1.2 Sex determination

Interspecies differences that may affect sensitivity and response to endocrine disrupters include quantitative and qualitative variability in endogenous hormone and receptor levels, differences in the duration and timing of critical periods of development, and interspecfic differences in the sex-determination process (Campbell & Hutchinson 1998).

Significant variability exists between wildlife species in the mechanisms that control sex determination. Sexual differentiation depends on chromosome constitution and is controlled by different hormones in different species. The genetic basis of sex determination in many fish species is largely unknown. However, present evidence indicates that a number of sex-determination mechanisms exist in fish, including polyfactorial systems, male and female heterogamy, hermaphroditism, and environmental sex determination (Bull 1983, in Campbell & Hutchinson 1998). Important differences in hormonal control of sexual differentiation exist between species since this process is primarily controlled by androgens in mammals, by androgens and estrogens in reptiles, and by estrogens in other vertebrates such as birds. Hence, the relative effects of exposure to endocrine disrupters during this stage of development may well be significantly different between species (Campbell & Hutchinson 1998).

Although little is understood about the sex-determination process in fish, the information available indicates that important species differences exist that should be considered before selection of a species for tests designed to detect endocrine-disrupting effects. For example, the critical period for sex-determination in salmonid fish is just a short period around the time of hatch. This has been shown by the fact that exposure to natural or synthetic estrogens during the few days around hatch will result in the fish all developing as functional females. Conversely, exposure to exogenous androgens during this period results in an all-male population (Donaldson 1986), and an inadequate dose of exogenous hormone can result in hermaphrodite fish. The critical window varies and is much longer (weeks) in some species, rendering them vulnerable to exposure for exogenous endocrine substances for longer periods of time (Campbell & Hutchinson 1998). Also, in contrast to salmonids, exposure of some fish species, such as channel catfish, to endogenous androgens during the critical period results in feminization (Campbell & Hutchinson 1998 for ref.).

In addition, some fish species are hermaphrodites and change sex naturally at some time of their lives. These differences are likely to result in differential sensitivities between species on exposure to endocrine disrupters at early stages of development, and also make it extremely difficult to make extrapolations from one fish species to another, and even more so between different classes of animals. To effectively study and understand effects of endocrine disrupters at critical stages of development , increased knowledge of the factors that affect sex determination in fish and, preferably the ability to distinghuish between sexes at early stages of development are required. Equally important is the ability to separate effects of xenoestrogens/androgens from natural climatic fluctuations such as temperature and light under field conditions.

3.2 Natural factors affecting reproduction and development: temperature

Both reproduction as well as larval development are especially sensitive to water temperature fluctuations. Several **in-vitro** and **in-vivo** studies have shown that changes in temperature affect the endocrine homeostasis during reproduction (Van Der Kraak & Pankhurst 1997), including changes in the secretion and physiological effects on hormones related to the hypophyseal-gonadal communication controlling the reproduction process. Considerably less studies have dealt with the long-term consequences of temperature changes on the reproductional cycle or the larval development. Even fewer studies have been done aiming at the study of the long-term ecological consequences of rising water temperature (Van Der Kraak & Pankhurst 1997).

The goal with the reproductive endocrine homeostasis is the production of viable gametes through a stepwise regulation of the gametogenesis, maturation, ovulation and spermiation and finally the spawning. As endocrine processes regulating these events are temperature dependent, it is not surprising that the processes as such are temperature sensitive. Gametogenesis is initiated by the differentiation of the gonadal germinal tissue to oogonia and spermatogonia which are transformed to oocytes and spermatocytes. Since the sexual differentiation is genetically controlled, temperature is one of the most important environmental factors in this regard. In some fish species, for example silver sides (*Atherinidae*), high temperature favours the development of males and lower temperature females. Another example is Tilapia (*Oreochromis niloticus*) in which high temperature is causing a dominance of males (Strüssman & Patino 1995 and Baroiller & Geraz in Van Der Kraak & Pankhurst 1997).

Gonadal development may also be affected by temperature, but this is highly variable between species. In salmonids, the gonadal development is mainly controlled by the photoperiod with temperature having less significance as long as it stays within physiological limits. In cyprinids, on the other hand, the effect of temperature on gamteogenesis is much more important. The early stages of gametogenesis demand lower temperature and increasing temperature excerts a stimulatory effect on the later stages of gametogenesis.

At the end of gametogenesis the final maturation of the gametes and the ovulation and spermiation must occur before the fish may spawn. In rainbow trout, ovulation takes place between 9-15 °C provided that the photoperiod is favorable. Final oocyte maturation and ovulation do not happen at temperatures below 9 °C and , despite that ovulation may take place at temperatures higher than 15 °C, roe quality will be low. At higher temperatures ovulation is unsuccessful in the rainbow trout and this is more dependent upon that ovulation or oocyte maturation rather than vitellogenesis are unsuccessful. High temperatures also affect male rainbow trout and this is mainly expressed as lower amount of milt.

Contrary to homeothermal organisms, the reproduction of which is taking place under constant temperature conditions, the reproduction of the poikilothermal fish is completely dependent on the external temperature. Temperature influences practically on every stages of reproduction, including gametogenesis, oocyte maturation, ovulation/spermiation, spawning and the consecutive larval development. This is also including the endocrine mechanisms during the reproduction, although they are largely presently not fully understood. However, it is known that especially coldwater adapted northern fish species must, in order to reproduce successfully, experience a combination of low water temperature and short day length. In this instance the sex hormones most probably are playing a vital role. Increased temperature for example during embryogenesis may have negative consequences due to premature hatch and by this are unable to find enough food.

3.3 Natural factors affecting reproduction and development: light period

Apart from temperature, light period is playing an important role at the reproduction of fish. Changes in day length together with varying temperatures seem to be important for the reproductional process in, for example, cyprinids (Vlaming 1975). However, it is not the light period per se that is important but the combination light period and temperature. In the spring spawning cyprinid (*Notemigonus crysoleus*), a short photoperiod together with high water temperature caused a gonadal regression. No regression was observed at lower water temperature (Vlaming 1975). In the same species, stimulated gonadal development was experimentally induced by the combination long photoperiod-high temperature regardless of season. A long photoperiod, or high temperature alone, were capable of inducing gonadal development. The experiments by Vlaming (1975) indicated that spermatogenesis and vitellogenesis were independent of external environmental conditions, but that spermiation and oocyte maturation depend on a combined long photoperiod and high temperature, and that they are connected with the secretion of hypophyseal gonadotropins.

Corresponding effects of photoperiod have also been observed in three-spined sticklebacks (*Gasterosteus aculeatus*) (Wootton 1984). The experiments with three-spined sticklebacks showed that the absolute length of the photoperiod was not decisive for the maturation process but the distribution of the daily light.

If fish are to avoid spawning under suboptimal periods of the year, they must be equipped with accurate mechanisms, allowing maturation and reproduction at the suitable season. Sticklebacks, like other fish species in boreal regions, exist in environments where light and temperature show large seasonal variation, and this variation is effectively applied at the control of the moment for the on-set and completion of reproduction.

Screening-tests for detecting endicrinologically active compounds

4

Because the normally functioning endocrine system consists of an extremely complicated co-operation between a number of different organs, enzymes, and messenger compounds, it is obvious that functional disturbances may occur on a number of different levels through effects on one or several components. Thus, the basic viewpoint must be that endocrine disrupters (ED´s) may have a very broad spectre of effect mechanisms.

Apart from that ED´s may have a direct estrogenic effects in a narrow sense, e.g. acting as pure xenoestrogens, they may affect animal or human reproduction in a number of other ways. For example, ED´s may act upon the enzymes controlling sex hormone metabolism, interact with receptors for other hormones than E2, or affect hormone producing organs such as the hypophysis or the adrenal cortex. Other possible mode of actions are that ED´s may affect the estradiol levels by indirect feed-back mechanisms.

Consequently it is a far from simple task to design suitable test systems for the identification of which exogenous substances have disrupting effects on the endocrine system. Generally, laboratory tests sofar developed have only embraced certain, those relatively specific parts of the endocrine system governing the reproductory processes in animals and man, whereas other aspects have been left unaccounted for.

On the other hand, epidemiological and ecoepidemiological methods have been used, where attempts have been made to connect a decreased reproductional success or certain diseases to some identified or suspected factor supposedly belonging to category endocrine disturbing substances. However, the large number of uncontrollable factors means that it is extremely difficult to make reliable conclusions on correlations between a specific substance and an endocrine effect based on these methods.

It is noteworthy that testing the reproductory toxicity of chemical substances for a long time has been included in the standardized testing protocols for testing new chemical compounds before their entering the market. At such tests it may be vindicated that they indirectly answer the question on whether a tested substance is estrogenic or not. However, these tests are designed in such a way that they mainly are directed towards detecting teratogenic or direct reproductory toxicity. They are not designed so that a weak estrogenic activity of a compound can be detected. Neither can use of these tests reveal so called late effects, where there is a lag-phase between exposure and manifestation of the effect.

Finally, it is generally not possible to establish synergistic effects between two or several simultaneously dosed endocrinologically active compounds by using these routine tests, including the whole or a major part of the reproductory cycle. The occurrence of such synergistic activation of the estrogen receptor, which has not been repeated by anyone, e.g. potentiation of the estrogenic effect at combined exposure with several xenoestrogens was indicated by Arnold et al. (1996).

For the reasons given above, it may not be considered as enough to regard estrogenicity or endocrine impact as parts of the reproductory toxicity only, and be satisfied with trying to detect such phenomena as parts of an integrated test for reproductory effects. It is necessary to focus upon the endocrine processes and develop testsystems providing possibilities to study responses at varying levels of the endocrine system. This is important since, as previously mentioned, xenoestrogens as well as other endocrine disruptors may affect several other functions than just reproduction.

For this reason, different experimental models have been proposed or developed for the testing of endocrine effects of exogenous compounds. The major part of the test methods have been concentrated specifically on the estrogenic function, e.g. on the screening of estrogenically active compounds including both estrogenic and anti-estrogenic ones. In its simplest version of the model the test substance is assumed to structurally or functionally mimic the endogenous hormone 17β-estradiol (E2) by binding to the estrogen receptor (ER) and through this cause a spectre of biological effects. This is thought to happen either so that the compound is producing a hormonal response, or that the substance is blocking the receptor, thus eliminating the normal hormonal function.

In order to increase the realism of the test systems McLachlan (1993) suggested that **in-vitro** testsystems should be developed where not only the binding capacity of the ligand to the receptor but also the receptor-ligand complexes interaction with the response element and gene activation are measured. Thus, it would be possible to screen substances with unknown endocrine toxicity for their function on receptor-mediated gene activation. McLachlan (1993) further suggested that a number of other receptors be included in a test system for the screening of the function of chemicals as endocrine disrupters. Apart from the ER, progesterone, androgen, glucocorticoid, retinoid, thyroxin and dioxin receptors should be included in the test system. With such a system, a base for a description of chemicals functional properties in this regard would be obtained and be described in a similar fashion as the solubility, melting point etc. of different substances are described.

4.1 In-vivo*-models- intact experimental animals*

4.1.1 Mammalian models

Within the framework for the investigations of the reasons for the clover disease, sheep were largely used as experimental organisms. In these investigations the growth of breasts of rams was used as indicator of estrogenic responses (Shutt 1976). These whole animal tests were necessary in order to obtain a picture of the mode of action of phytoestrogens, their metabolism in the intestinal tract, and their distribution and break-down after uptake in the blood circulation.

Intact animal models have also been utilized for the study of the effect mechanisms and the development of so called late effects, for example at studies on if exposure for a substance during the embryological development or during the post-natal state is producing reproductory effects in the adult mature organism. For example, effects on the development of sperm producing Sertoli cells in male rat or mouse testicles can be studied after exposure to a test substance from late embryogenesis up to 15-18 days post-birth, e.g. the period when Sertoli cells normally are formed. This kind of model studies may answer the question if low but chronic exposure to xenoestrogens during sensitive developmental stages may cause weak but significant effects on the reproductive capacity later in the life of organisms.

This field of research is presently under rapid development and despite that the number of more or less specific test-methods already is significant, there is a continuous increase of emerging methods, making the field hard to grasp by the non-specialist.

It is important in this instance to remember that estrogenic compounds may induce several separate classes of responses via independent mechanisms, where different types of estrogen receptors are involved. The main responses are usually separated into three categories (Bustos et al. 1996):

1. Those mechanisms that depend on hormonal stimulation of the genome through hormone-interaction with receptors in the cytosol or cell nuclei in different types of uterus cells that are expressed as increased RNA or protein synthesis in the uterus cells (Jensen and DeSombre 1972)
2. Mechanisms that are independent of the genome and are induced through hormone-interaction with so called ″eosinophilic leucocyte-estrogen receptors″ and are expressed as uterus edema, increased permeability of blood vessels or secretion of histamine.
3. Other types of responses including for example membrane-bound estrogen receptors in the uterus, cyclic AMP and prostaglandins (Bustos et al. 1996).

As noted, there are multiple and independent mechanisms for estrogenic effects that are controlled by the physiological status of the test organism. Estrogenic compounds may furthermore interact selectively with certain receptorsystems in certain cells, but not in other ones, making the picture all but clear. Consequently, it is important to recognise both the occurrence of different mechanisms, as well as that possibilities presently exist to register a broad spectre of responses when choosing test systems and performing systematic testing of estrogenic effects.

4.1.2 Fish models

Traditionally reproduction tests with various fish species, where the exposure for the test substance has taken place from the adult stage to early larval stages, or during the whole life cycle, have been used for the identification of substances with toxic effects on some part of the reproductory cycle. However, by the use of such ″black-box″ tests it has usually been difficult to discriminate between estrogenic or otherwise endocrinologically active substances from substances affecting reproduction via other effect mechanisms.

The possibility to develop more specific methods for the identification of exogenous compounds interacting with the hormonal regulation in fish was opened by the finding that steroids affect growth, sex determination and various reproductory processes in fish (Callard et al. 1980; Frostier et al. 1983).

Already in the 1970s it was shown that the liver of rainbow trout was stimulated to produce vitellogenin by estrogen and other steroids (Clemens 1978; Chen 1983; Maitre et al. 1985). Vitellogenin is a high molecular weight glycolipophosphoprotein actin as a precursor at the synthesis of egg yolk protein in all egg laying vertebrates. Vitellogenin is normally produced in female livers upon estrogen stimulation and is thereupon transported in the blood to the ovary, where it is incorporated into the growing oocytes. Inside the oocytes the vitellogenin is transformed into the two most important egg yolk proteins, lipovitellin and phosphovitin. These proteins form the most important nutrient reserve in the egg (and yolk sac) and act as food for the fish embryos until they start actively feeding themselves.

In male fish no, or very low levels of vitellogenin can be detected. However, exposure of male fish to estrogenic compounds is inducing the vitellogenin synthesizing gene, resulting in elevated dose-response related levels of vitellogenin in the blood of male fish (Bulger & Kupfer 1983).

These observations have later emanated in vitellogenin measurements both **in-vivo** laboratory and field experiments as well as **in-vitro** laboratory tests from the blood of male fish, and this method seems to be one of the most effective methods used for determination of exposure of fish to estrogenic compounds (Pelissero & Sumpter 1992).

A number of different methods for the determination of the vitellogenin levels in fish have been developed and adapted for different fish species, for example carp (Tyler & Sumpter 1990) and flounder (Pereira et al. 1992).

Lately the vitellogenin test has been completed with a parallel measurement of the so called ″zona radiata″ proteins (zrp), which also are induced by estrogenic substances. It has been shown that zrp-measurement is a still more sensitive method for the detection of estrogenic compounds and zrp has therefore been suggested as a supreme biomarker for estrogenicity (Arukwe & Goksöyr 1996).

Also some other physiological changes have been noted in fish exposed to estrogenic compounds. Some of these changes may be supposed to be so highly specific that they may be used as biomarkers for the identification of estrogenic or otherwise hormonlike compounds, while other responses are much less specific. For example, it has been shown that E2 in rainbow trout is inducing synthesis of steroid binding protein (SBP) (Foucher et al. 1992) as well as zrp (Hyllner et al. 1991), both of which are critical for the reproduction of fish. It has also been shown that phytoestrogens can both stimulate synthesis of SBP and interact with SBP so that the binding between endogenous steroids and SBP is negatively affected (Pelissero & Sumpter 1992).

Other, less specific effects of high E2 doses administered via feed to rainbow trout are hypertophy and lipidemia of liver and kidney cells (Herman & Kincaid 1988). Similar effects were obtained when siberian sturgeon was fed a diet containing E2, whereas the effects disappeared when this diet was changed to a steroid free diet (Pelissero et al. 1991).

Another fish model, suggested by Bortone and Davis (1994) as a potential test system for detection of hormonally active water borne compounds, is based on the fact that females of certain viviparous mosquitofish species may show changed external sexual characteristics and mating behaviour (masculinization) at exposure to exogenous sterols (Denton et al. 1985; Krotzer 1990).

4.2 In-vitro *tests*

Two complementary aspects may be studied at **in-vitro** tests: proliferation of an estrogen-dependent cell-line and induction of an estrogen controlled function. Additionally, pure receptor-binding tests may be added to this group.

4.2.1 Binding to receptors in cells

The principle behind receptor-binding tests, regarding ER, is to try to release radioactively labelled E2 from ER, by applying increasing concentrations of either unlabelled E2 (the reference curve) or varying kinds of purified substances.

The biological material used is either a total extract or an extract of cell nuclei from a tissue containing high concentrations of ER, for example rabbit or rat uterus or MCF-7 (a human breast cancer cell culture). Another possibility is to use a cytosol-extract of fish liver rich in ER-binding sites (Jobling et al. 1995).

A corresponding methodology may be used at testing the binding affinity to other specific hormone receptors or the Ah (dioxin)-receptor. For example, hormonal effects of contaminants in male animals can be simulated by testing the binding to the androgen receptor (AR) by using rat prostate cytosol (Kelce et al. 1995). Moreover, methodology has been developed for the testing of the egg-membrane-bound receptor for the maturation inducing steroid 17α,20,β21-trihydroxy-4-pregnen-3-on in fish (Thomas et al. 1996).

4.2.2 Proliferation of cancer cells

At these tests, estrogen-dependent cancer cell cultures are used. The today most frequently used **in-vitro** tests related to mammalian animal systems is the so called "E-screen" based on the MCF-7 cell culture, having been in use since the 1970s. The test is based on the dose-response relationship between the proliferation of the MCF-7 cells and the E2 dose that the cells are exposed to during a 6-day period. The number cells or the amount of incorporated radioactively labelled thymidin are used as variables for the measurement. By comparing the effect of a xenoestrogen with the effect of E2, the relative estrogenic potential of the test substance is obtained (Soto et al. 1994).

MCF-7 cells have also been used to measure the progesteron-receptor induction parallel with the measurement of the ^{3}H-thymidin-incorporation, thus enabling testing of the combined effect on cell proliferation and functional change by the test substance.

A further development of the E-screen test is that so called reporter plasmids with DNA-sequences coding for genes with specific and easily measured gene products are incorporated into MCF-7 cells. By this it is can be determined if the estrogenic compounds directly are stimulating ER´s transcription activity (Jobling et al. 1995).

Several different human cell lines can be used as **in-vitro** cell culture test systems. Apart from MCF-7, also the cell lines ZR-75 and T-47D , isolated from breast cancer cells, can be used for testing of the cell proliferation velocity under exposure to suspected xenoestrogens (Jobling et al. 1995).

4.2.3 Vitellogenin screening test

As already mentioned above, isolated rainbow trout hepatocytes have been used for vitellogenin synthesis measurements induced by exposing the cell culture for xenoestrogens. This test system, developed by Jobling and Sumpter (1993), and used for rapid screening of the existence of estrogenic compounds in community sewage effluents, have lately been increasingly used by scientist as a rapid and effective testing tool.

4.2.4 Recombinant yeast screening test

Since it was shown that human ER is functional in yeast cells (Metzger et al. 1988), several groups of scientists developed parallel rapid, sensitive and simple screening tests based on yeast cells (*Saccharomyces cerevisiae*) (Klein et al. 1994; Routledge & Sumpter 1996). Yeast does not contain sex steroid or thyroid hormone receptors, but nevertheless homologous proteins to those that are needed for activated transcription in mammalian cells. Therefore the DNA sequence corresponding to the human estrogen receptor has been integrated with one of the yeast cells chromosomes by gene technology. The yeast cells also contain plasmids carrying the reporter-gene lac-Z (coding for the β-galactosidase enzyme), which is used to measure the activity of the receptor. At a positive response β-galactosidase is excreted to the external medium and may be easily detected spectrophotometrically by a colour change.

This yeast screening test has appeared to be considerably more sensitive than alternative **in-vitro** tests and has been increasingly used for rapid screening of a number of suspected xenoestrogens.

Hormonally active phytoestrogens and their sources

5

5.1 Isoflavonoids

5.1.1 Occurrence and turnover

Of the substances in plants that may have hormonal effects in animals , the isoflavonoids attain a special position through their extensive occurrence, their relatively high concentrations in plants, and their, in many cases, conspicuous endocrine activity.

In plant tissues the isoflavaonoids occur as sugar derivatives, glycosides, and their concentrations vary greatly depending on the extent of stress the plant is suffering from, for example in the form of viral-, fungal-, or bacterial attack or through grazing (Barrett 1996).

The most extensively studied plant species regarding occurrence and turnover of isoflavonoids is soy. Already in 1931 it was noted that soy beans contained high levels (1-3 mg/g) of glycoside of two isoflavaonoids, namely daidzein and genistein (Walz 1931; Eldridge & Kwolek 1983). Later, low levels of some other isoflavonoids (glycitein, coumesterol, pratensin, prunetin) have been identified in addition to the main two components mentioned above.

Vegetables also contain high levels of isoflavonoids and they have also been identified in the body fluids of man (Barrett 1996). In the human intestine isoflavonids are hydrolyzed forming so called aglycones, which are either absorbed from the intestine, excreted or transformed. Following phytoestrogens belonging to the isoflavonoids have been identified from human urine by Adlercreutz et al. (1991):

- formonetin
- daidzein
- dihydrodaidzein
- methylequol
- genistein
- 3´,7-dihydroxyisoflavan
- equol

Recently also glycitein and five metabolites was identified in human urine (Kelly et al. 1993). The structure and transformation pathways of some isoflavonoids is given in Figure 5.

Following metabolic transformations are known (Barrett 1996):

- Formonetin → Daidzein → Equol
  O-desmethylangolensin
- Biochanin A → Genistein → p-ethylphenol (hormonally inert)

At least the transformation of daidzein to equol has been shown to be influenced by the intestinal microflora of several warm blooded animal species (Setchell et al. 1984).

In sheep, breakdown of biochanin A and genistein is induced only after several days intake of isoflavonoid-rich feed (Verdeal & Ryan 1979).

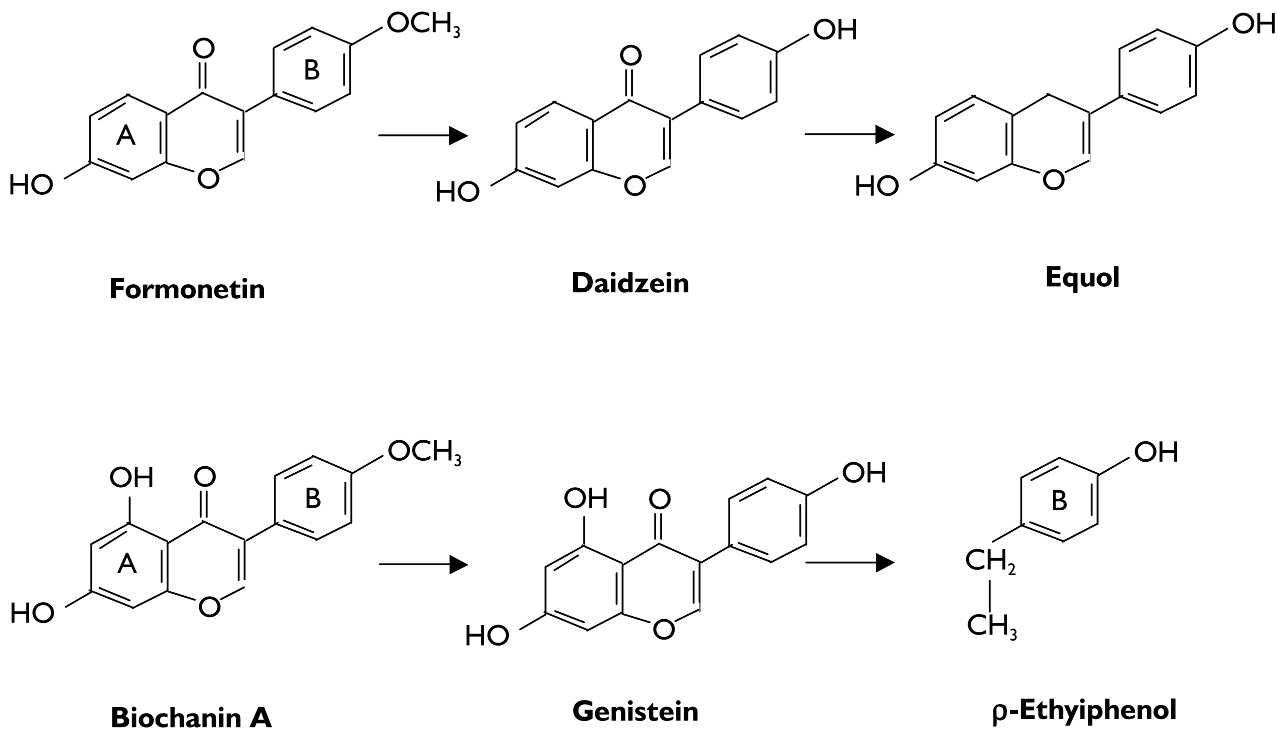

Figure 5. Chemical structure and transformation pathways of some isoflavonoids.

As previously touched upon, the levels of isoflavonoids in plant tissue is highly influenced by fungal and bacterial attacks. Hence , the isoflavonoids are suspected to possess antibacterial and antifungal properties. The free isoflavonoids have a stronger fungistatic effect than their corresponding glycocides and it is possible that a glucocidase from the attacking fungus may be responsible for the activation of the plant´s isoflavonoid synthesis (Verdeal & Ryan 1979). Interestingly in this instance is the observation that soy leaves damaged by ozone accumulate daidzein (Keen & Taylor 1975), leading to the somewhat speculative hypothesis that increasing levels of air pollutants, mainly ozone, could result in increasing levels of isoflavonoids in plants and feedstock.

5.1.2 Examples of effects on animals

In a very extensive number of **in-vitro** and **in-vivo** studies, it has been shown that isoflavonoids can produce clear endocrine and other biological effects in animal systems (see Adlercreutz 1995 and Barrett 1996). Similarly, isoflavonoid-rich diet produces various biological effects in animals, including fish and humans.

Besides that the previously mentioned isoflavonoids (genistein, biochanin A, daidzein and equol) show a clear affinity for the estrogen receptor (ER), they have been shown to have both estrogenic and anti-estrogenic effects in a number of test systems. Both daidzein and equol show a strong affinity for ”nuclear type II estrogen binding sites” in the rat uterus and are by this supposed to affect the regulation of estrogen-stimulated growth.

Genistein is a known inhibitor of thyrosine-specific protein kinases and of DNA-topoisomerase II and protein histidine kinase (Adlercreutz 1995). Thyrosine kinases are thought to play a role in the cell proliferation and transformation and their activity is connected to the action of the breast cancer oncogene. It is assumed that the broad biological activity shown by genistein may explain this compound´s capacity of inhibiting the growth of cancer cells in both estrogen dependent MCF-7 and estrogen independent MDA-468 breast cancer cell cultures (Barrett 1996).

Both genistein and daidzein stimulate synthesis of the sex hormone binding protein (SBP) in the liver, which indirectly affects the levels of free sex hormones (testosterone and E2) in the body. This results in a reduction of the normal metabolic breakdown of steroids as well as reduction of their normal biological activity (Adlercreutz 1995).

In studies using Siberian sturgeon as test organism it was found that biochanin A, daidzein, equol and genistein could induce vitellogenin synthesis in juvenile fish (Pelissero & Sumpter 1992). The strongest effect in the fish was obtained with genistein, namely an effect that was 2 000 times lower than that of E2. The disturbance between the binding of endogenous steroids and the SBP as well as stimulated SBP synthesis has been observed also in fish.

Despite the uncertainty as to what the consequences of induced synthesis of vitellogenin may be for juvenile male fish, it has been assumed that the heavy metabolic strain caused by the vitellogenin synthesis may result in negative impact on growth, disease resistance and reproduction (Pelissero & Sumpter 1992). Stimulation of SBP is supposed to more directly influence upon the reproduction of fish.

5.2 Coumestans

5.2.1 Occurrence and turnover

Coumestans are structurally similar to isoflavonoids and up to 20 different naturally occurring isomers in different plant species have been reported (Wong 1975). Among these only two have been shown to be estrogenically active, e.g. coumestrol and 4´-methoxycoumestrol (Figure 6). These substances mainly occur in alfalfa and in the clover species *Trifolium repens,* but have also been detected in relatively high concentrations in several food stuffs (Verdeal & Ryan 1979).Additionally, one coumestan, psoralidin, isolated from steppe grasses from Central Asia has on good grounds been suspected to be estrogenic (Moule et al. 1963).

The concentrations of coumestrol in feedstuffs (beans, peas, soy products) are usually around or below 1 µg/g dry weight, but for example in fresh soy sprouts concentrations higher than 70 µg/g have been detected (Knuckles et al. 1976).

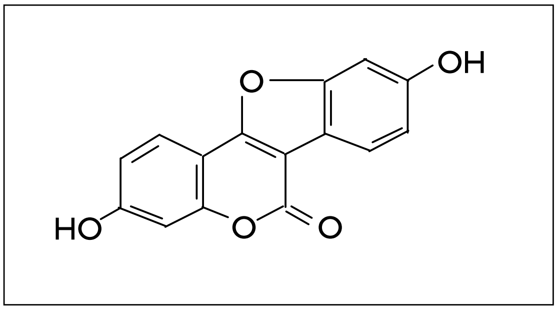

Figure 6. Structural formula of coumestrol.

Similarly as the isoflavonoids, coumestrol synthesis seems to be induced in the plant by insect and fungal attacks. Concentrations as high as 2.4 mg/g have been documented from the leaves of heavily infested alfalfa (Verdeal & Ryan 1979).

5.2.2 Examples of effects on animals

Coumestrol has ER-mediated as well as other estrogenic effects in a number of different test systems. The effect pattern is pretty similar to that obtained at testing genistein, but in most cases coumestrol exhibits a stronger estrogenic effect than genistein and other isoflavonoids (Mäkelä et al. 1994)

More recent studies, however, have shown that the effect of coumestrol probably is produced through a considerably more complicated way than previously assumed. For example it is possible that the estrogen response to coumestrol (and other phytoestrogens) is largely dependent on the level of endogenous estrogen in the test organisms (Markaverich et al. 1995). Therefore, it has been concluded that coumsetrol is to be regarded as an atypical estrogenic substance, above all since it did not produce increased DNA levels in uterus of the test animals.

Fish treated with coumestrol produced a relatively strong induction of vitellogenin synthesis, coumestrol being 4-5 times more effective than genistein. Thus, relative the effect of E2 the effect of coumestrol was only 500 times lower (Pelissero & Sumpter 1992).

5.3 Flavonoids

5.3.1 Occurrence and turnover

The flavonoids belong to a group of naturally occurring plant pigments that are daily consumed by humans in gram quantities, since they are ingredients in common vegetabilic food stuffs. For example following compounds, of which the structural formulae are given in Figure 7, belong to the flavonoid group:

- quercetin
- resperetin
- narigenin
- luteolin

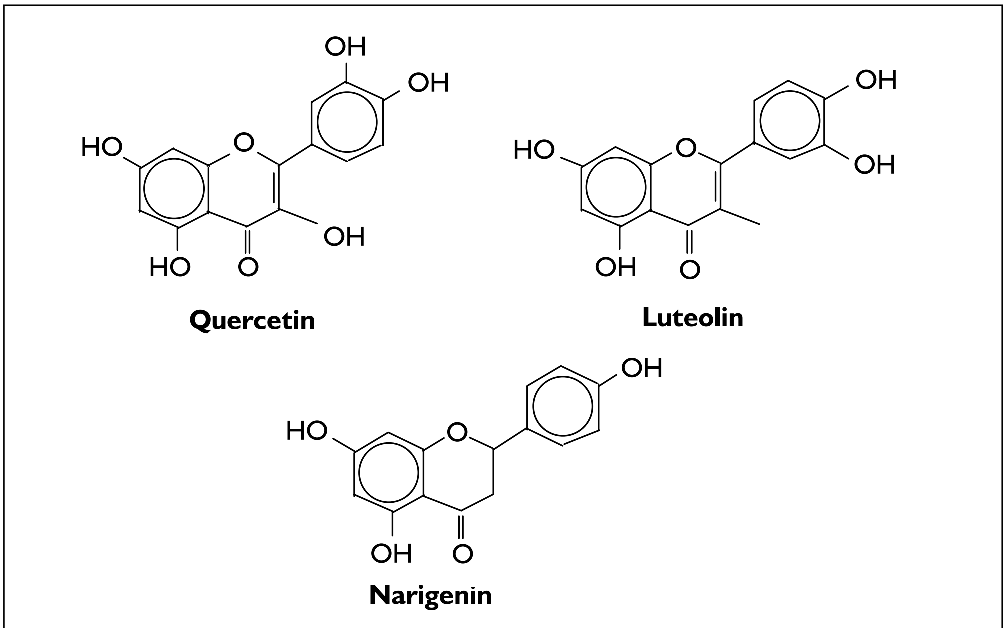

Figure 7. Examples on flavonoid structure: quercetin, luteolin and narigenin.

5.3.2 Examples of effects on animals

The hormone-related effect mechanism of the flavonoids obviously deviates from the effect mechanism of the estrogenic compounds sofar discussed (Markarevich et al. 1995). The flavonoids do not bind to ER, but instead to so called nuclear type II E2 binding sites. A stimulation of these sites is a characteristic prerequisite for estrogenic stimulation of DNA synthesis in the cells as well as in cancer cells.

At least quercetin and luteolin show a high affinity to nuclear type II E2 binding sites, but not to ER, and this binding is correlated with an antagonistic effect on the estrogenic response in the rat uterus (the substances inhibit E2-induced stimulation of the uterus weight) and with inhibition of several types of cancer cells **in-vitro** (Markaverich et al. 1995). The naturally occurring flavonoid metabolite methyl-p-hydroxyphenylacetate has been identified as an endogenous cell growth regulator and as the natural ligand for type II sites. These observations have led to the conclusion that flavonoids (consumed via diet) and their metabolites profoundly may affect reproductory processes in mammals as well as the formation of estrogen-related breats - and prostate cancer.

The relative contribution from flavonoids to the total intake of estrogenically active substances in normal-diet humans has been calculated by Safe (1995). Based on the relative estrogenic potential of flavonoids, 0.1-0.2 % of the E2 potential (Miksicek 1993), and on a daily calculated intake of 1.02 g/day, an estimated daily intake as estrogen equivalents of flavonoids of about 100 μg/day was obtained. This intake was estimated to be about 160 times lower than the intake through contraceptive pills (Safe 1995).

No studies on how this kind of substances affect the endocrine system of fish have been found.

5.4 Lignans

5.4.1 Occurrence and turnover

Lignans are phenolic compounds consisting of two phenylpropane units, connected at the β-carbon atom in the side chain. They are assumed to be synthesized via coniferylalcohol. Common lignans occurring in grain flour, plant fibres and linseed as well as in a number of fruits and vegetables are (Figure 8, Barrett 1996) :

- secoisolariciresinol
- matairesinol

Both compounds have been found in rye- and wheat flour and they seem to be attached to the aleuronic layer in the grain seed (Adlercreutz 1995). Other lignans are lariciresinol isolariciresinol, which beside the other two, are found in human urine.

Usually lignans are divided into plant lignans and ”animal lignans”. The latter constitute diphenols with lignan structure, but are lacking an oxygen atom from the para-position and are assumed to be metabolic products of plant lignans, formed microbiologically in the intestinal tract (Adlercreutz 1995). The most essential mammalian lignans are enterodiol and enterolactone. These two compounds were first identified in 1979 in human urine (Setchell et al. 1980). In addition enterodiol may be further oxidized and form enterolactone (Setchell & Adlercreutz 1988). Especially high enterolactone levels have been found in human sperm and cattle (Dehennin et al. 1982).

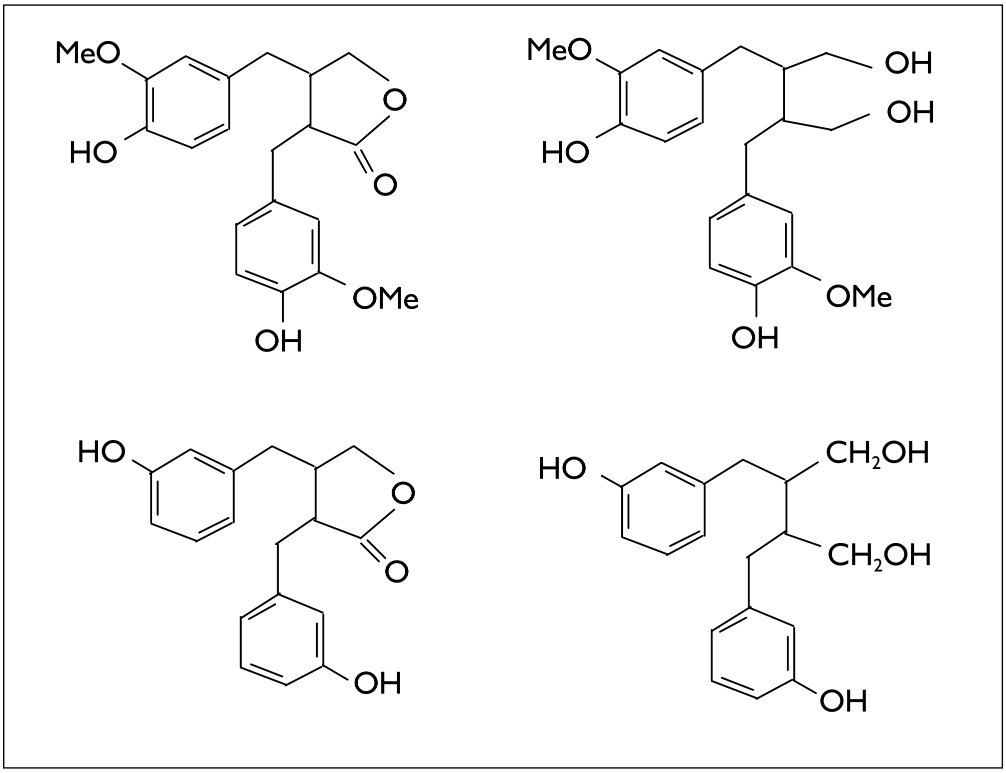

Figure 8. Examples on lignan structures: uppermost the plant lignans matairesinol and secoisolariciresinol, below the "mammalian lignans" enterolacton and enterodiol.

Contrary to the estrogenic isoflavonoids and coumestans, that sofar have not been identified in tree species used as raw material for pulp production, several lignans with suspected or shown estrogenic properties occur in common tree species in Scandinavia (Mellanen et al. 1996).

The total lignan concentration in heartwood of 75 year old spruce, *Picea abies*, has been measured at 1 mg/g (Jörgensen et al. 1995) levels, and the major components and their concentrations were:

- hydroxymatairesinol 390 µg/g
- allohydroxymatairesinol 210 µg/g
- lyovil 116 µg/g
- matairesinol 85 µg/g
- secoisolariciresinol 70 µg/g

According to earlier studies the total lignan level in spruce wood may be up to 0.5 % based on the wood dry weight (Ekman 1979).

It has also been shown that hydroxymatairesinol can be transformed to a-conodreicacid (Ekman & Holmbom 1989), a compound occurring at relatively high levels in the effluents from thermomechanical pulp production using spruce as raw material (Jörgensen et al. 1995).

Worthwhile to mention is also that spruce wood contains relatively high levels of the two lignans matairesinol and secoisolariciresinol, considered to be precursors of the two mammalian lignans eneterolactone and enterodiol. However, to date no reports on their occurrence in effluents from pulp mills have been found.

5.4.2 Examples of effects on animals

The transformed lignans enterodiol and enterolactone have a weak affinity to the rat uterus cytosol, but no estrogenic effects of these substances have been detected in mice **in-vivo** (Adlercreutz 1995). However, it has been possible to show estrogenic effects of these two lignans in four sensitive **in-vitro** test systems, whereas no anti-estrogen effects have been observed. For instance, it has been found that enterlactone stimulates the pS2-gene in MCF-7-cells. In a **in-vivo** study using rat as test organism enterolactone was found to inhibit the estrogen-stimulated RNA synthesis in uterus tissue. Moreover, lignans have been shown to stimulate SBP synthesis in liver, inhibit the aromatase enzyme and affect the secretion of gonadotropins (ref. in Adlercreutz 1995). Wood lignans are effective inhibitors of aromatase in human placenta, although their estrogenic influence has not yet been shown **in-vitro** (Mellanen et al. 1996).

All in all, at least the two transformation products of plant lignans, eneterodiol and enterolactone, may be regarded as weak endocrine disruptors with a broad spectre of effects, including estrogenic ones.

5.5 Mycotoxins

5.5.1 Occurrence and turnover

A group of estrogenically active substances occurring in animal feed and human vegetabilic food products are the recorcyloxylactones zearalenone (Figure 3) and its derivatives. These compounds are not naturally produced plant components, but metabolites synthesized by the plant infecting fungus *Fusarium roseum.* These compounds are commonly connected to the group ″mycotoxins″ (Price & Fenwick 1985). Zearalenone is also usually regarded as a ″fermentation estrogenic substance″ or F-2 toxin.

Fusarium-infection of different agro-products (wheat, barley, corn) is a very common phenomenon.. It has been shown that of all wheat and corn stored in American grain storehouses about 10-20 % are contaminated with zearalenone with concentrations up to 10 mg/kg (Verdeal & Ryan 1979). Zearalenone has been identified from several food stuffs including beer (4-5 mg/kg) above all in countries with poor grain storage conditions (Price & Fenwick 1985).

5.5.2 Examples of effects on animals

Zearalenone and some of its derivatives belong to the most potent natural estrogens having an estrogenic potency of 0.1-10 % of E2, depending of the kind of test used (Verdeal & Ryan 1979; Price & Fenwick 1985). According to more recent tests performed with MCF-7 cellcultures the relative estrogenic effect of zearalenone was measured to 3-5 % of E2, whereas coumestrol obtained a relative value 0.03 % and genistein 0.01 % (Mayr et al. 1992).

Due to the relatively strong estrogenic effect of zearalenone, and due to its extensive occurrence in important food stuffs, considerable research on this substance has been done. For example work has been devoted to developing methods for detoxification of zearalenone-contaminated grain.

A synthetic zearalenone derivative has been patented and has been used as a growth hormone in beef production (Mäkelä et al. 1994). Its estrogenic potential is up to 7 times higher than that of zearalenone (Verdeal & Ryan 1979).

The effect pattern obtained with zearalenone with different test systems for the measurement of endocrine impact are largely similar to those obtained at exposure with isoflavonoids and coumestrol. However, some fundamental differences have also been documented. For example, zearalenone as opposed to genistein and coumestrol, did not inhibit the reduction of estrone to E2 in T-47D cell cultures (Mäkelä et al. 1994). In general, it has been noted that zearalenone as genistein and coumestrol, is acting through an ER-mediated mechanism as a relatively potent xenoestrogen. The substance stimulates cell growth in uterus, and more specifically the synthesis of DNA and RNA and proteins. Moreover, zearalenone hampers development of ovaries, adrenals and pituitary in test organism, possibly by inhibiting gonadotropins (Martin et al. 1978). Zearalenone is also reported to induce congenital defects.

5.6 Stilbenes

5.6.1 Occurrence and turnover

Stilbenes are naturally occurring substances embracing both compounds with a genuine stilbene structure (C6-C2-C6) as well as compounds with a structure closely resembling the stilbene structure. The main groups consist of genuine stilbenes (1,2-diphenylether), bibenzyls (diphenylethanes), 1,2-diphenylethanols and phenyl-dihydroisocoumarins (Figure 9).

Most naturally occurring stilbenes are polyhydroxy-substituted compounds. In plants they occur in free phenolic form, as ethers, usually methylethers, and as glycopropanoids (Mannila 1993). The most common natural stilbenes are pinosylvin with its methylethers and following compounds, which partly occur as free phenols, partly as their respective glycopyranoids:

Phenolic form	**Glycopyranoid form**
Resveratrol	Piceid
Piceatannol	Astringin
Isorapontigenin	Isorapontin

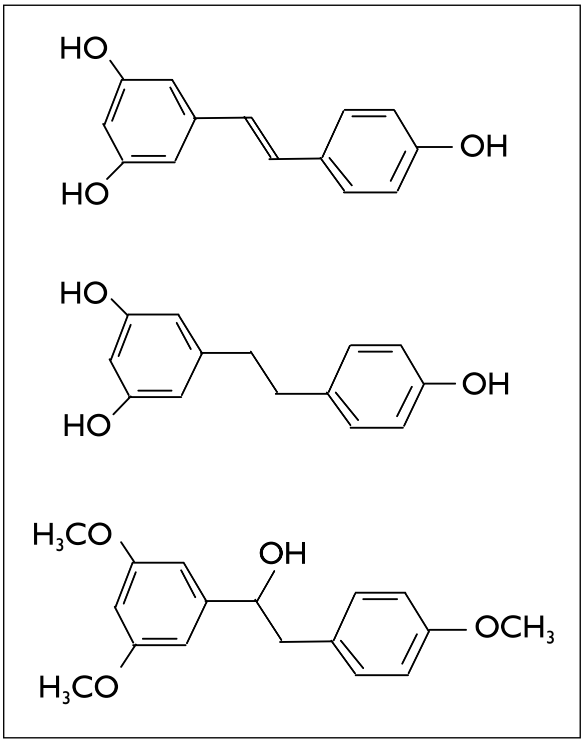

Figure 9. Main groups of stilbenes: 1,2-diphenylethene, 1,2-diphenylethane, and 1,2-diphenylethanol (lowermost).

Relatively high levels of stilbenes have been identified in softwood, mainly in the bark and the needles, but the concentration varies with season, age and environment (Mannila 1993).

Many other plants also contain stilbenes, not least the tropical- subtropical bush- and tree family *Combretaceae,* which healing properties (for example plague and cancer) during a long time has been taken advantage of in African and Indian folk medicine. (Watt & Breyer-Brandwijk 1962). However, stilbenes seem to occur in phanerogams since no reports of their identification in cryptogams or bacteria exist (Mannila 1993).

The most common natural stilbene, pinosylvin, occurs in the wood of the pine tree. The molecule contains two hydroxyl groups in the same aromatic ring, making it less probable of pinosylvin being estrogenic. However, in the bark of different spruce species several polyhydroxystilbenes are occurring:

- resveratrol
- piceatannol
- isorapontigenin

Together with their respective glycosids the total amount of these stilbenes in the bark of spruce was 1-6 % of the dry weight (Mannila & Talvitie 1992).

The structure of the most common stilbene derivatives are presented in Figure 10.

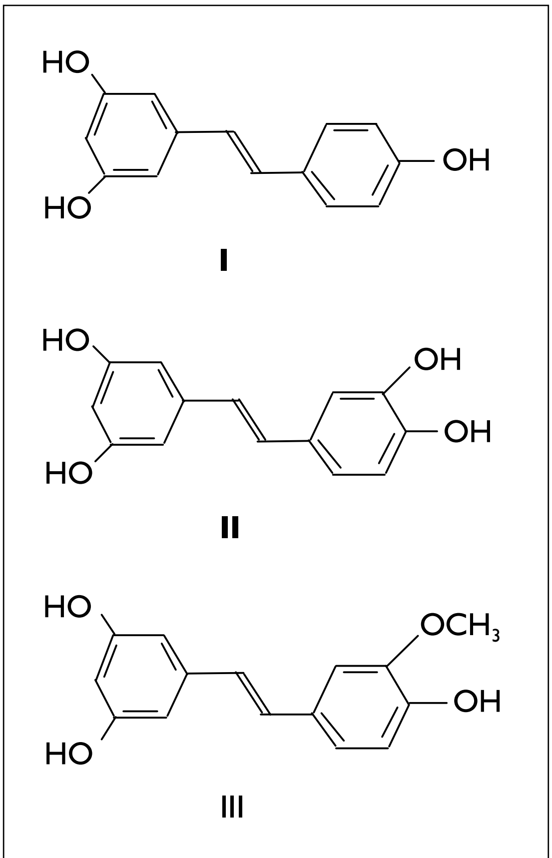

Figure 10. The most common stilbene derivatives:
I - reveratrol;
II - piceatannol;
III - isorapontigenin.

In the tree tissues, the physiologically most important role of the stilbene derivatives is assumed to be the protection against fungal and insect attack. For example the fungicidal effect of pinosylvin is well known. Although pinosylvin normally occurs in the main wood component of the tree, it may also be found in more superficial wood layers after fungal or insect attack, mechanical damage, dehydration or at exposure to ethylene (Mannila 1993). It has also been observed that the stilbene levels in bark and needles in different softwood species is increasing conspicuously as the trees are exposed to sulphur dioxide and/or ozone.

Naturally occurring stilbenes are not only active against plant pests and pathogens. In traditional medicine plant extracts assumed to contain large amounts of stilbenes are used for the treatment of a number of ailments such as rheumatism, artherosclerosis and malaria (Mannila 1993).

5.6.2 Examples of effects on animals

In his work on biologically active stilbene derivatives Mannila (1993) refers to a large number studies dealing with varying kinds of biological effect of these substances. Below, only results dealing with the potential of stilbenes to produce estrogenic effects are referred to, however.

When the estrogenic activity of piceatannol, isorapontigenin and astringin was tested **in-vitro** using MCF-7 cell cultures only weak responses were observed. **In-vivo** mice tests did not produce any estrogenic or anti-estrogenic results on any of the substances tested (Mannila 1993).

However, more recent tests with isorapontigenin, isorapontin, pinosylvin, piceatannol and resveratrol have shown that this group of compounds are estrogenically active. In cancer cell growth tests **in-vitro** with T-47D cell strain it was seen that some of the compounds tested were estrogenic, isorapontigenin and isorapontin having the highest estrogenic potential. Pinosylvin had a considerably lower estrogenic potential and piceatannol and resveratrol lacked estrogenic potential in this test (Mellanen et al. 1996).

When testing the capacity of pinosylvin to induce synthesis of vitellogenin mRNA in rainbow trout after intraperitoneal injection, no response was obtained (Mellanen et al. 1996).

Although the hitherto reported studies on the stilbenederivatives endocrine activity sofar may be regarded as ambiguous, indications exist that at least some components naturally occurring in softwood may be estrogenically active. In this instance further research is warranted in order to more closely reveal under what conditions and to what extent the endrocine potential is manifested in different test systems as well as under natural conditions.

5.7 Indole-3-carbinol

5.7.1 Occurrence and turnover

Indole-3-carbinol (I3C) and related compounds (Figure 11) occur amply in varying vegetables belonging to the *Brassicales* family, above all in cabbage, cauliflower and broccoli. Interest in this compound and its metabolites, mainly indole-[3,2-b] - carbazol (ICZ) strongly increased when their capacity as inducers of Ah-receptor mediated responses in mammalian cells as well as their anti-estrogenic effects and breast cancer cell growth inhibition capacity was revealed (Liu et al. 1994; Safe 1995).

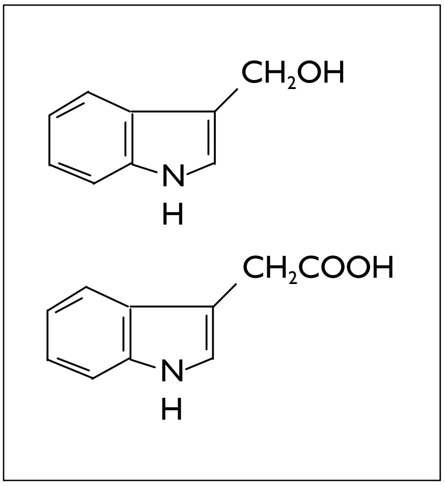

Figure 11. Structural formula of indole-3-carbinol and the plant hormone indole-3-acetic acid.

The closely related compound indole-3-acetic acid is an important plant growth hormone.

It is known that I3C is transformed irreversibly to its dimer 3,3´-di-indolylmethane and to polymers in the stomach of mammals. Both I3C and the dimer are powerful inducers of cytochrome-P450 mono-oxygenases and of estradiol-2-hydroxylase in rat liver, whereby the dimer has been shown to have the strongest potential (Jellink et al. 1993).

Safe (1995) has, based on the relative anti-estrogenic potential of ICZ of 0.1 % of the potential of TCDD, calculated the daily human intake of TCDD-anti-estrogen equivalents via I3C metabolites to 0.25-1.3 ng/day, which is about 10 times more than the corresponding intake via TCDD and chloroorganic compounds.

5.7.2 Examples of effects on animals

It has been noted that increased consumption of cabbage varieties correlated with reduced tumour frequency in humans (Young & Wolf 1988) and test organisms (Stoewsand et al. 1988).

Closer analysis on the mode-of action showed that I3C binds to specific cytochrome P-450 enzymes in the liver, possibly through a mechanism involving the Ah-receptor. These cytochromes have the capacity of increasing the 2-hydroxylation of endogenous estrogens on the account of formation of 16-α-hydroxysteron, and thereby protecting the target cells from this harmful steroid. 16-α-hydroxysteron has namely been shown to act as a cancer initiator in mammals and also significantly diminish the ER- and progesteron-receptor levels in MCF-7 cell cultures (Jellinck et al. 1993).

The metabolite ICZ, like many other substances with strong affinity for the Ah-receptor (e.g. TCDD) has a clear antiestrogenic effect on human breast cancer cells. ICZ is also weakly estrogenic in MCF-7 cells and binds to ER (Liu et al. 1994).

5.8 Resin acids

5.8.1 Occurrence and turnover

Resin acids occur within the so called resin channels as well as within resin pockets in the wood and the bark of softwood. They do not occur in hardwood. Resin acids are biosynthesized by the epthelial cells lining the resin channels.

At the digestion of sulphate softwood kraft pulp the resin acids are almost completely dissolved in the alcaline cooking liquor as sodium soaps and are finally washed out from the pulp during the pulp wash. At the evaporation of the combined black liquor and washing liquid the resin soaps are separated together with fatty acid soaps and lipophilic neutral compounds (e.g. sterols) in the form of so called sulphate soap or tall oil soap. The soap is skimmed off and cooked with acid to form so called crude tall oil. The tall oil consists of about 40-50 % of resin acids when digesting pine wood. Digestion of wood from spruce produces only half as much tall oil as pine wood digestion, and it contains a lower amount of resin acids (Holmbom & Ekman 1978).

In order to improve the wash-out of hydrophobic, neutral extractives from birch and aspen, tall oil is usually added to the digestion. Therefore resin acids also occur in effluents from the production hardwood pulp.

Remains of resin acids from the pulp wash end up in the effluents from mills producing sulphate kraft pulp due to black liquor spills and via minor discharges of effluents from the wash stage, bleach plant effluents, condensates and debarking effluents.

At the production of mechanical and chemimechanical pulps the resin acids are dispersed in the process waters together with lipids occurring in the parenchymatic cells of the wood. At pH<6, generally prevailing at production of mechanical defibration in TMP-, SGW-, and PGW, all lipophilic extractives occur together as colloidal droplets with a size of 0.1-2 µm (Ekman et al. 1989). Very little of the resin and fatty acids are dissolved in the water phase. At pH>6 the resin and fatty acids are dissolved from the droplets and occur mainly as dissolved soaps in the water phase. This is the case at the production of CTMP pulp (pH 8-10) and at the bleaching of mechanical pulp using alkaline peroxide (pH 9-11). When acidifying these pulps, the resin and fatty acids return into the colloidal droplets.

Among the most common resin acids present in the soft wood furnish used in Scandinavian pulp mills, and consequently also in the effluents discharges, may be noted dehydroabietic acid, abietic acid, neoabietic acid, isopimaric acid and palustric acid. Recently published data from New Zealand (Tavendale et al. 1996) show that other quantitatively important components in the resin acid complex may be abietanic acid, 13-abietenic acid, piamric acid and seco-1- and seco-2-abietic acid.

The kinds of transformation- and break-down products that are formed from resin acids under biological treatment, or when they are subjected to the natural microbial community in the receiving waters, are poorly known. It is generally assumed that resin acids are relatively easily degradable, but the fact that a number of resin acids, among them dehydroabietic acid, can be traced in the sediments from pulp mill effluent receiving waters, is speaking against such a view. For example Tavendale et al. (1996) showed that resin acids were not appreciably broken down in the sediment during a 40-year period of time.

In a recently published investigation by Stuthridge and Tavendale (1996) it was observed that resin acids were not broken down to any significant extent in an aerated lagoon in New Zealand. Similar result were obtained by Ånäs et al. (1982). Biotransformation of resin acids occurred in an anaerobic zone at the inlet of the aerated lagoon. Among the transformation products, decarboxylated resin acids (hydrocarbons)., partly or completely hydrated resin acids and oxidized resin acids were found. However, scientists from New Zealand have shown that resin acids under anaerobic conditions may be transformed to neutral compounds belonging to the group polyaromatic hydrocarbons, such as fichtelite, dehydroabietin, retene and tetrahydroretene, which have been detected in the sediments of effluent receiving waters (Judd et al. 1996).

On the other hand, in a Finnish study an about 85 % break-down of the resin acids was noted in an activated sludge treatment plant without formation of detectable levels of biotransformation products (Huhtiniemi et al. 1996). Less than 1 % of the in-coming resin acids to the treatment plant remained in the treated effluent. The major part of the non-degraded resin acids went to combustion together with the sludge.

Also Carey et al. (1995) have, referring to Wilkins and Panadam (1987), pointed out that anaerobic transformation of resin acids may result in the formation of retene and pimarene. These last mentioned polyaromatic hydrocarbons are known to be strong inducers of the Cytochrome P-450 system, for example the EROD-enzyme. Retene (Figure 12) has been found in 1 000-2 000 mg/g levels in sediment close Canadian mill effluent discharge points, whereas the retene levels in sediments from uncontaminated areas were at the 1 µg/g level (Carey 1996).

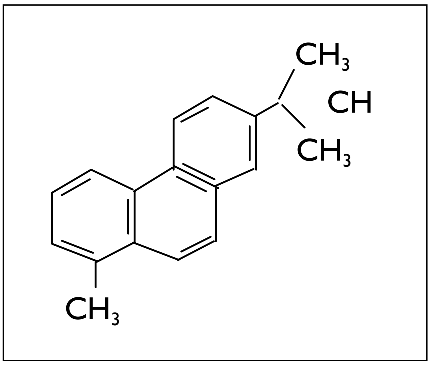

Figure 12. Structural formula of retene.

5.8.2 Examples of effects on animals

It has since a long time ago been known that resin acids are acutely toxic to fish, but it was only recently that a Finnish group of scientists obtained indications on the possibility that an abietic acid extract may produce estrogenic effects when tested on T-47D cell cultures (Mellanen et al. 1996).

However, upon control the abietic acid extract was found to contain only 37 % abietic acid (and 6 % dehydroabietic acid), whereas the remaining components remained unidentified. Consequently, it was not unequivocally proven that specifically the abietic acid was the compound responsible for the relatively strong estrogenic potential noted at the test (a weak vitellogenin induction was also obtained when rainbow trout was fed the same preparation). In fact, the authors cautioned not to draw definitive conclusions on the estrogenic effects of abietic acid at the present state.

In this connection, it is worthwhile mentioning recently published data by Carey (1996) on the capability of retene as a strong inducer of the EROD-enzyme. However, it is presently not possible to make any conclusions on whether retene also shows affinity for the Ah-receptor and/or shows estrogenic or anti-estrogenic properties in adequate test systems.

Altogether, from the data given above, it may be supposed that the resin acids (or abietic acid) do not necessarily belong to substances that specifically have estrogenic effects in tests systems. It cannot be excluded that the resin acid preparation used may have contained transformation products, that may have been the estrogenically active compounds. Moreover, there are reasons to suspect that formation of strong cytochrome P-450 inducers may occur in external treatment plants and/or in receiving waters into which resin acids are discharged.

5.9 Phytosterols (Triterpenoids)

5.9.1 Occurrence and turnover

Sterols are important components in both animal, fungal and plant cell membranes and they are thought to be regulators of the fluidity, stability and permeability of cell membranes. Some sterol molecules are also assumed to have non-structural, metabolic functions (Schuler et al. 1990; Fischer & Höll 1991).

Pine and spruce wood mainly contain sitosterol, sitostanol and campesterol in additon to the dimethylsterols cycloartenol and metylenecycloartenol (Holmbom & Ekman 1978). Birch wood contains, in addition to the before mentioned sterols, considerable amounts of the methylsterol citrostadienol (Bergman et al. 1965; Holmbom & Avela 1971) and the pentacyclic triterpenealcohol lupeol. Betulinol occurs in high amounts, ca. 25 %, in the outer bark layer of the birch tree (Ekman 1983). Other important components in the bark of birch is methylbetulinate and lupeol as well as triterpene alcohols with oleane-structure (erythrodiol, methyoleanoate etc.). Wood of aspen contains, in addition to sitosterol, cycloartenol, methylenecycloartenol and citrostadienol, also butyrospermol (Lindgren & Svahn 1966; Sjödahl et al. 1985, unpubl.). Especially in aspen wood the pentacyclic triterpenealcohols α-amyrine and β-amyrine occur in higher levels than the sterols.

The chemical structure of some important compounds are given in Figure 13.

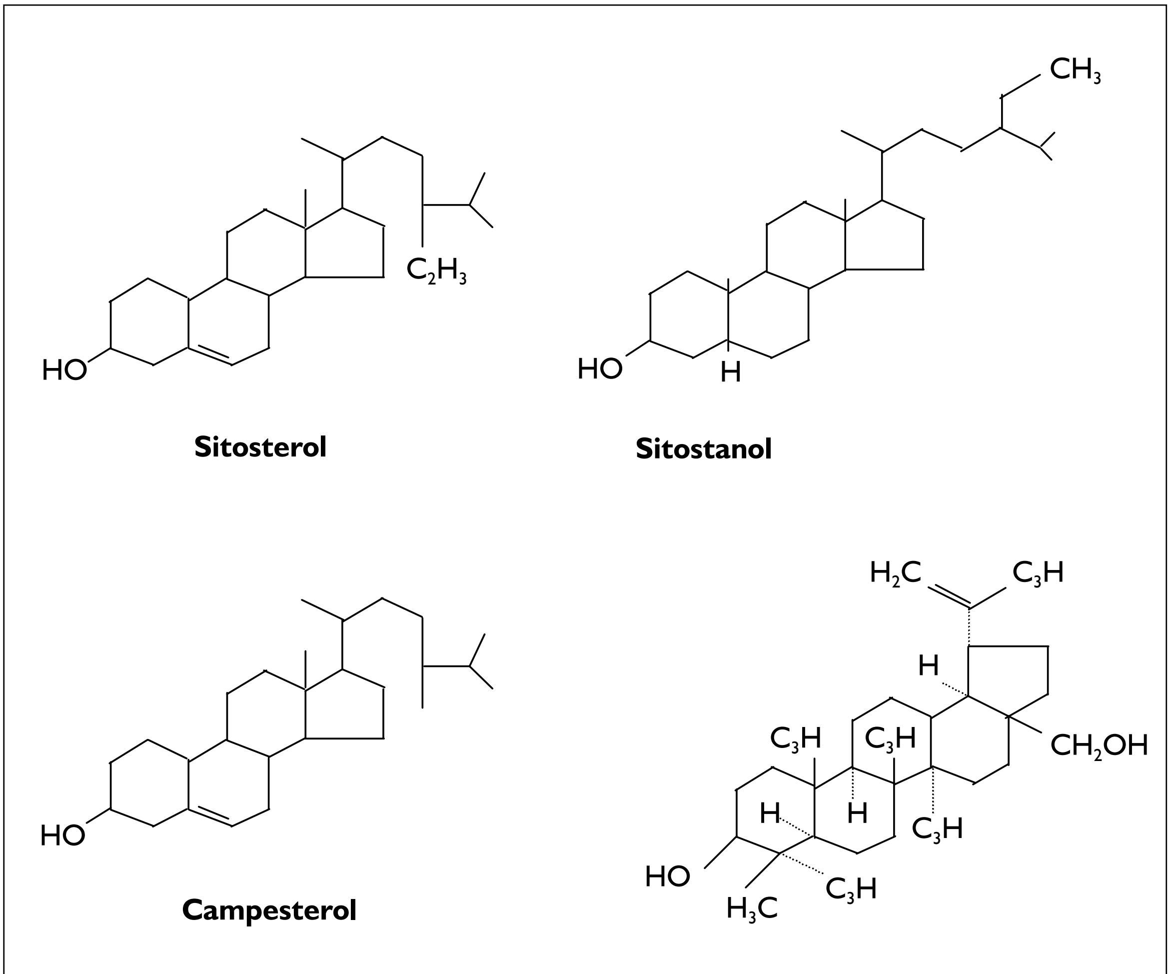

Figure 13. Examples on phytosterols (sitosterol, campesterol and sitostanol and triterpene alcohol betulinol.

Also other plant species (herbs) than trees frequently contain complex mixtures of phytosterols, of which the dominating ones are sitosterol, stigmasterol, and campesterol (Schuler et al. 1990). Especially soy-products form an important raw material for the production of phytosterols (Hise 1983).

At the production of sulphate kraft pulp, the phytosterols and the triterpenealcohols are separated from the wood fibres toghether with the resin and fatty acids, and end up in the tall oil. The amount of the tall oil as well as its chemical composition vary according to the geographical origin and the age of the wood. However, it has been shown that neither the total amount, nor the extractable fatty acid composition of sterylesthers, vary with season (Fischer & Höll 1991). Crude tall oils from Norwegian pulp mills contain 4-11 % sterols and triterpenealcohols (Holmbom 1978; Holmbom & Ekman 1978). The highest amounts of sterols are found in talloils with a high proportion of birch in the process and the lowest amounts in tall oils from pulp production using high proportions of spruce. In crude tall oils the amounts of sitosterol are 2-6 % and those of sitostanol 0.4-1-1 %.

It is sofar unclear what kind of transformation products that are formed under microbial processing of phytosterols for example in pulp mill industrial external effluent treatment plants. However, it is well known that some strains of the bacterium *Mycobacterium smegmatis* have the ability to transform a mixture of sitosterol and sitostanol to substances with endocrine effects in fish (Denton et al. 1985). Also, at the production of pharmacologically useful products of sitosterol, mutant strains of *Mycobacterium* are utilized for the cleavage of the saturated alcyl-sidechain from the sterol molecule for the production of corticosteroids and spironolactone (Hise 1983).

Although it is generally observed that phytosterols present in pulp mill effluents are biochemically degradable, and thereby are reduced at biological effluent treatment, these substances can still be detected in treated effluents. At a Finnish newsprint pulp mill (mainly TMP) equipped with an activated sludge treatment plant the sterols were less degraded than the resin acids (Huhtiniemi et al. 1996). The major part of the sterols were, however, removed with the biosludge that was sent for combustion. Only about 10 % of the sterols in the incoming effluent left the effluent treatment plant. The amount of sterols in the treated effluent was, based on tons produced product, 22 and 46 g at two sampling occasions.

In a study of the discharges from three pulp and paper mills in SE Finland during the spring 1995 the total levels of sterols and triterpenealcohols were 0.09-1.2 mg/l and the emissions were 10-85 kg/d corresponding with 7-92 g/t pulp (including chemical and mechanical pulp) (Wikström et al. 1997). The total levels of phytosterols and triterpenealcohols in treated effluent from modern pulping processes (ECF and TCF) have been determined at 0.2-18 g/t pulp.

At a Finnish sulphate pulp mill producing bleached hardwood and softwood pulp, effluent analysis was performed before and after a major modernization including change from conventional chlorine bleaching to ECF bleaching and replacement of the aerated lagoon with an activated sludge treatment plant (Oikari and Holmbom 1996). Despite of a more than 60 % decrease of the COD and a 95 % reduction of the resin acids in the treated effluent, it was shown that the levels of sitosterol and betulinol remained approximately at same level as before the modernization. More recent studies have shown that the level of total phytosterols showed an about 96 % reduction in the treatment plant (Tana et al. 1999).

5.9.2 Examples of effects on animals

It is a long known fact that the androstene derivatives that are producable through fermentation of phytosterols by the use of certain *Mycobacterium*-strains are important raw materials for the production of medicines, predominantly corticosteroids, but also certain other androgens, estrogens and progesterons (Hise 1983). Hence, it is well established that various transformation products of phytosterols possess a strong hormonal activity. Whether such hormonally active metabolites are produced in appreciable amounts in external treatment plants and/or in natural receiving water bodies is presently not well known.

To date, only a few reports exist on the endocrinological activity of more or less pure phytosterols on mammalian cells. In a recent study such experiments were performed with sitosterol (>91 % purity), sitostanol (>90 % purity), citrostadienol (65.7 % purity) and betulinol (>50 % purity) on **in-vitro** cell proliferation using MCF-7 and/or T-47D as test models (Mellanen et al. 1996). The results showed that sitosterol was active in the T-47D test but not in the MCF-7 test, sitostanol was active in T-47D-cells, betulinol was active in MCF-7-cells, whereas citrostadienol was inactive. Betulinol showed the strongest estrogenicity. As sitosterol, apart from its inactivity in the MCF-7 test, also was inactive in a few other tests, the authors reached the conclusion that this particular substance was not estrogenic in itself, but needs metabolic transformation in order to become hormonally active (Mellanen et al. 1996).

The same group of scientists also made tests with the three hormonally active phytosterols´ capacity to induce the vitellogenin gene in juvenile rainbow trout. A very distinct vitellogenin-response was obtained both with oral feeding of sitosterol and by intraperitoneal injection of the same substance, whereas neither sitostanol nor betulinol showed any activity in this test (Mellanen et al. 1996). The clear estrogenic activity of sitosterol is somewhat confusing, since earlier studies on this , as well as a closely related phytosterol after metabolization above all showed androgen activity in different test systems.

For example, it was shown that a mixture of 65 % sitostanol and 30 % sitosterol, incubated in the presence of *Mycobacterium smegmatis* , caused a masculinization of female mosquitofish (*Gambusia affinis)* together with a typical masculine mating behaviour (Denton et al. 1985; Krotzer 1990).

At a long-term exposure of three-spined stickleback to sitosterol via water, a growth stimulation was recorded, and at exposure of rainbow trout via feed and water for the same compound, decreased liver glycogen levels and inhibition of the liver conjugation enzyme UDP-GT (Lehtinen et al. 1993).

Recently a connection between exposure to phytosterols and effects on reproduction and brood of fish was reported (Lehtinen et al. 1999). Effects on egg survival, increased congenital deformities in hatched fry, lower weight at hatch, and stimulated growth after swim-up were noted. The effects were mediated through the maternal part of the fish, since cross-fertilization with unexposed males and exposed females produced the same response as when both genders were exposed and fertilized. A dose-dependent increase of phytosterols in the roe suggests that phytosterols tend to accumulate in the roe of fish. The lowest concentration tested was 10 µg/l producing effects mentioned above indicating that the no effect level would be lower than 10 µg/l of phytosterols.

Other experiments have also revealed a hormonal activity of sitosterol in exposed fish. Upon injection of sitosterol in goldfish of both sexes, a significantly depressed level of plasma-testosterone and 11-ketotestosterone in male fish as well as depressed levels of testosterone and E2 in female fish were noted by MacLatchy and Van Der Kraak (1995). Simultaneously, the gonadotropin-levels increased in male goldfish. The results suggest that sitosterol has the capacity of reducing gonadal steroid synthesis and also that phytosterols may contribute to the reproductional disturbances noted in fish from pulp mill effluent receiving waters.

Despite that the effect pattern observed at exposure of the dominating sterol, sitosterol, on various test systems, is hard to explain and even controversial, there are clear indications on hormonal activity of this substance. Further research is needed in order to clarify if sitosterol in its original or in metabolized form is active, if its main effect is androgen or estrogen, and against what functions of the endocrine system the compound (and its metabolites) is acting upon.

6 Hormonal effects of effluents from pulp and paper industry

Chronic impacts on the aquatic ecosystems are usually considered as the most undesirable ecotoxicological effects. A group of substances that have attracted special interest over the last years is the so-called endocrine disrupting substances, because they may inflict chronic effects on living organisms by interacting with their natural hormone system and thereby disturb essential life functions such as growth and reproduction. Pulp mill effluents have been implicated to be a possible source of endocrine disrupting chemicals. Two different experimental approaches have been used in studies of the possible significance of hormonally active substances in pulp mill effluents. A large array of laboratory test methods have been developed and used. Various **in vitro** tests use cell cultures for rapid screening of estrogenic responses, while **in vivo** tests with intact test animals usually offer a more realistic test situation, and therefore provide more reliable results. The second approach has been to study reproductive responses in fish under field conditions in pulp mill effluent receiving waters. The considerable improvements of the environmental standard of the mills allow the existence of fish populations in the vicinity of modern mills. This, in turn, makes it possible to undertake detailed investigations of feral fish in areas close to the discharge points in order to assess whether or not residual waste materials cause minor, albeit unacceptable, effects on the long-term sustainability of sensitive species and populations.

This overview will mainly focus on fish models for testing the endocrine activity of water-borne chemical compounds and various effluents.

6.1 Identification of endocrine disrupting activity

Following parameters and endpoints have been used in identification of endocrine disrupting activity.

Vitellogenin. Already in the 1970s it was demonstrated that the liver of rainbow trout can be induced to produce vitellogenin (VTG) by treatment with estradiol and some other steroids (Clemens 1978). VTG is the protein precursor of yolk and is synthesised by the liver exclusively in response to estrogens. In sexually immature fish and in males there is almost no natural estradiol, so VTG induction in these fish is a good marker of exposure to exogenous estrogenic substances. Induction of VTG is mediated in through specific binding of the exo-estrogen to the estrogen receptor (ER), and the level of induction can be determined by measuring the concentration of VTG in the blood plasma of the fish. The induction of VTG in male rainbow trout following estrogen treatment has been correlated with decreased testicular growth, which is generally accepted as a negative reproductive consequence (Jobling et al. 1996).

Plasma steroid concentrations. In contrast to VTG induction, alterations in plasma sex steroid concentrations can result from several different mechanisms of action, including direct effects on steroidogenic enzymes or indirect modifications associated with altered feedback loops (Ankley et al. 1998). For example, an estrogen antagonist could interfere with the feedback inhibition of steroidal synthesis, leading to increased steroidogenesis in the ovaries. Decreases in plasma androgens have been shown to result from exposure to 17-β estradiol in several fish species, presumably due to feedback inhibition of androgen synthesis (Jobling et al. 1996).

Receptor binding. The interaction of xenobiotics with hepatic ER has been assessed in several fish species, while considerable uncertainty exists in the identification of chemicals that interact with the fish androgen receptor (AR),because relatively little work has been done with this receptor. It is still under debate whether or not to use other receptors unique to fish, such as the membrane-bound progestogen and the gonadotropin receptors in the gonads, oocytes and sperm, in the screening of EDs (Ankley et al. 1998).

Alterations in secondary sexual characteristics. Such alterations have been used as endpoints indicative of endocrine disruption in studies with pulp mill effluents mainly from North-America (Bortone & Davies 1994).

Alteration in sexual differentiation. Although sexual differentiation can serve as a potential indicator of ER or AR agonists, various factors make this endpoint uncertain. For example, the differentiation mechanism in different fish species is uncertain in that outcomes are not always predictable. Moreover, environmental factors such as water temperature can affect sex determination and ratios in fish (Ankley et al. 1998).

Gonadosomatic index (GSI). The ratio of gonad weight to body weight is a reasonable screening endpoint in terms of indicating (anti-) estrogenig/androgenic chemicals because it is easily measured and applicable to both sexes of oviparous fish species. The GSI is highly integrative in that it is not necessarily specific for any particular mechanism of action, but can reflect, for example, circulating hormone levels. Changes in GSI also can be indicative of likely reproduction success and, hence, population-level effects. However, as is true for steroid levels, such an endpoint should only be compared in fish that are at the same stage of gametogenesis (Ankley et al. 1998).

Steroidogenesis. As is the case for mammalian systems different researchers have assessed gonadal_steroidogenesis in fish both **in vitro** and **ex vivo** protocols. For example, gonads of wild fish collected from rivers receiving pulp and paper mill effluents were shown to have diminished steroid-producing capacity. In addition, the steroidogenic capacity of isolated ovarian follicles of fish has been shown to be significantly altered when exposed to xenobiotics **in vitro** (Ankley et al. 1998).

Final gamete maturation. This endpoint involves collecting eggs and sperm from exposed fish and evaluating gamete quality and maturation at the completion of vitellogenic growth and spermatogenesis. Like the GSI, final gamete maturation is a broadly integrative endpoint that reflects a variety of mechanisms affected by EDs and is generally applicable to all fish species. Gamete size, germinal vesicle breakdown (GVBD), ovulation, hydration, and lipid and oil droplet formation all are relatively easy endpoints to assess using basic light microscopy (Ankley et al. 1998).

Germinal vesicle breakdown. In order to determine if a xenobiotic is capable of inhibiting maturation of oocytes, an in vitro assessment of GVBD (the final maturation of the eggs) can be performed in a culture system that includes the chemical of interest, oocytes removed from their follicles, and maturational gonadotropin. Chemicals that inhibit GVBD probably interact with the membrane steroid receptors for maturation-inducing hormones (Ankley et al. 1998).

Hypotalamic-pituitary-gonadal axis. Endogenous hormones and environmental chemicals can affect reproductive function by acting at a number of sites within the hypothalamic-pituitary-gonadal axis. A further complication is that chemicals could affect independent pathways (pituitary hormone secretion, gonadal steroid hormone secretion, altered peripheral steroid metabolism) yet lead to a reduction of plasma steroid hormone levels. Furthermore, even in cases where chemicals exert their effects on a common target or response, there are often multiple pathways controlling specific processes. This underlines the need for detailed studies defining the precise mechanisms by which complex mixtures and pure compounds affect reproductive function in order to establish cause and effect relationships (Van Der Kraak et al. 1998b).

Induction of hepatic mixed function oxygenase (MFO) activity. This test is by no means specific for endocrine modulating substances, but some EDs (as well as some natural sex steroids) do have a high capacity for inducing this enzyme system, usually assayed by measuring the activity of ethoxyresorufin-O-deethylase (EROD) in fish liver. The MFO system contains a group of cytochrome P-450 enzymes that catalyze many types of reactions that are primarily oxidative and facilitate the excretion of molecules from the organism. Exogenous molecules with specific structures such as coplanar rings, e.g. "dioxins" (TCDD) and polyaromatic hydrocarbons (PAHs) are believed to activate the P-4501A protein through the aryl hydrocarbon (Ah) receptor in cells and have the potential to simultaneously cause a variety of deleterious effects either directly or indirectly related to the Ah receptor (Martel et al. 1997).

In terms of screening for chemicals or effluents with (anti-) estrogenic/androgenic properties, the present information on the endocrine system of other aquatic organisms, such as reptiles and amphibians, is insufficient to make direct comparisons to other vertabrate classes. Thus, how predictive screens and tests from other species will be for these animal classes is unclear.

Direct and indirect evidence suggest that invertebrates are sensitive to chemicals in the environment that disrupt endocrine systems: however, from the perspective of screening for (anti-) estrogenic/androgenic effects, enough uncertainty exists concerning the role of these systems in invertebrates making it premature to recommend specific tests/endpoints. However, from the standpoint of existing tests, it is suggested that sexually reproductive stages be considered in the chronic reproductive tests to increase sensitivity to EDs that cause reproductive and developmental toxicity (Ankley et al. 1998).

6.2 Reproductive responses in fish observed in field studies in pulp mill effluent receiving waters

Observations of alterations in such life functions that are known to be under hormonal control, such as growth and reproduction, will give an indication of the extent to which endocrine modulating substances are present in the effluents and still appear in sufficiently high concentrations after dilution in the receiving waters to induce a detectable impact on the feral fish populations. Alterations in endocrine homeostasis and reproductive fitness have been reported in a variety of feral fish populations exposed to pulp mill effluents (Table 3).

The earliest observations of impacts on the reproductive system in feral fish being exposed to pulp mill effluents seem to be those made in Floride, USA, where masculinised mosquitofish (*Ganbusia affinis holbrooki*) were found at a number of sites in two streams (Howell et al. 1980). The masculinization effect involved the development of male secondary sex morphological structures, in particular the modification of the anal fin into a gonopodium-like structure. Behavioural changes were also noted. The changes were found downstream, but not upstream, of a kraft pulp and paper mills, and have therefore been linked to such effluents. This causal relationship was later confirmed by laboratory studies (Drysdale &

Bortone 1989). It was also noted that the two streams in Florida represented a worst-case scenario, as the flow of the streams was small, suggesting that the phenomenon is perhaps seen only at high concentrations of mill effluents.

Perhaps the most extensive among early field studies evaluating the endocrine and reproductive responses of fish to pulp mill effluents were those on white sucker (C*atostomus commersonii*) exposed to bleached kraft mill effluents (BKME) in Jackfish Bay, Lake Superior. These fish exhibited a wide variety of altered repro-

Table 3. Selected examples of reproductive and development abnormalities observed with pulp and paper mill effluents.

Species	Site/Laboratory test	Observations	Contaminant	Reference
Mosquitofish	River, Florida	Masculinization of female fish, development of male secondary sex morph.structures	BKME	Howell et al. 1980
Whitefish	Lake Päijänne	Lower occurrence of larvae, lower reproductive success	Paper mill effluent	Hakkari & Bagge 1992
White sucker	St Maurice River	Reduced plasma hormonal levels	BKME	Gagnon et al. 1994
Fathead minnow	Laboratory, lifecycle test	Delayed spawning, reduced egg production, depressed secondary sexual characteristics	BKME	Robinson et al. 1994
White sucker	Jackfish Bay	Reduced steroid biosynthetic activity followed by disruptions in precnecolorae formation	BKME	McMaster et al. 1995
White sucker	St Maurice River	Greater length at maturity, reduced gonad size more variable fecundity	BKME	Gagnon et al. 1995
Fathead minnow	Laboratory, lifecycle test	Impacts on time to maturation, egg production and gender	Different BKME	Kovacs et al. 1995a
Fathead minnow	Laboratory, lifecycle test	No significant deviation from control up to 20 % effluent concentration	TMP	Kovacs et al. 1995b
Different species	Bothnia bay/ Baltic Sea	Increased age at maturation, reduced gonad size increased growth	BK.	Sandstorm et al. 1996
Goldfish	Laboratory	Reduced in vitro steroid production, not detected after mill modifications	BK.	McMaster at al. 1996b
White sucker	Jackfish Bay	Delayed sexual maturation, reduced fecundity at age, reduced plasma sex steroid levels, decreased gonad size	BKME	Munkittrick et al. 1992
White sucker	Jackfish Bay	Elevated apoptatic DNA fragmentation in ovarian follicular cells	BKME	Janz et al. 1997
White sucker	Moose River	Reduced ovarian size and steroid levels	BKME/TMP	Munkittrick et al. 1998
Mummichog	Miramichi estuary	Delayed gonadal maturation, reduced egg size	BKME	Leblanc et al. 1997
Fathead minnow	Laboratory, lifecycle	Depressed sex steroid production, delay in sexual maturity, reduced egg production	BKME	Van Der Kraak, 1998
White sucker	St Maurice River	Increase of size at first maturity	BKME	Bussiere et al. 1998
Rainbow trout, juvenile	Laboratory exposure	Elevated plasma vitellogenin, no effects on testosterone levels, lreduced pregnenolone levels	BKME	Tremblay & Van Der Kraak 1999
Whitefish, juvenile	Lake Saimaa	Increased vitellogenin gene expression	BKME	Mellanen et al. 1999
Brown trout	Laboratory exposure	Reduced testosterone levels, lower hatching success in untreated effluent, no effect in treated effluent, slower growth in larvae fromexposed parents	TMP, untreated and secondary treated effluents	Johnsen et al. 2000

ductive responses, that were thought to indicate exposure to endocrine modulating substances, i.e. delayed sexual maturation, reduced fecundity with age (in females), decreased gonadal size, reduced expression of secondary sexual characteristics (in males), reduced plasma sex steroid levels and plasma gonadotropin-II levels (Munkittrick et al. 1991, McMaster et al. 1996a, Van Der Kraak et al. 1998b). Despite the above mentioned reproductive responses, exposure to BKME did not affect fertility of eggs and sperm obtained from fish reaching sexual maturity nor subsequent larval development (McMaster et al. 1992). Depressed steroid levels or increased vitellogenin gene expression have also been demonstrated in fish at other mills (Hodson et al. 1992, Servos et al. 1992, Gagnon et al. 1994) and in other species at other sites (Adams et al. 1992, Munkittrick et al. 1994, Soimasuo 1997, Mellanen et al. 1999, Karels 2000).

It was also found that the consequences of alterations in steroid hormone levels to whole animal reproductive fitness varied greatly between fish species. For example, BKME exposure did not affect gonadal size in the longnose sucker (*C. catostomus*). In contrast, lake whitefish (*Coregonus clupeaformis*) from Jackfish bay had limited ovarian and testicular development and >90 % of the fish were not developing gonads for the upcoming spawning season (Munkittrick et al. 1992). Same kind of observations of gonadal disorders have been reported in burbot (*Lota lota*) on the northern coast of the Bothnian Bay, Finland. The observations recorded in these studies lead to the conclusion that sterility occurs in adult burbot in some places affected by effluents from pulp mills and that this cannot be regarded as a temporary phenomenon in the reproductive biology of the species (Pulliainen et al. 1999). In another study from Finland the annual reproductive success of vendace (*Coregonus albula*) and whitefish (*C. lavaretus*) in some polluted and clean areas of the central part of Lake Päijänne was estimated in 1981-1990 on the basis of occurrence of larvae in shore seine and seine net samples. Larvae of coregonids were found only occasionally in the vicinity of a paper mill (0-5 km) and further away (5-15 km) the density of larvae was decreased as compared to clean areas. In spite of the improvement of the water quality in the area during the 1980s, the reproductive success of coregonids was still low. The reasons for this were assumed to depend on adverse oxygen conditions of the hypolimnion and sediment and the toxic effects of the effluents (Hakkari & Bagge 1992). While these observations provide evidence for differences in responsiveness to BKME or paper mill effluents, the factors contributing to these differences are still unknown.

Field studies in Sweden in the 1980s demonstrated impacts on body growth and reproduction of fish exposed to pulp mill effluents (Sandström et al. 1988). Although there have been a variety of physiological parameters used to evaluate performance of fish, the ones reflecting reproductive function may be the most useful. Continued field work in Sweden and Canada have provided data base allowing assessment of the frequency of reproductive effects in fish populations from various pulp mill sites. Sandström (1996) reviewed a number of field studies and found that age at maturity was delayed in more than 75 % of pulp mill studies reporting it, and gonad size was depressed in almost 60 % of the studies, whereas growth rate was increased in <30 % of the studies and depressed in <10 %. Furthermore, in studies where growth rate was increased, maturity was also delayed in all studies where it was also measured concomitantly (Sandström 1996). The changes in reproductive steroid hormone levels appeared to offer the best potential for predicting the potential of effluents to affect maturity, egg production, and secondary sex characteristics. Detailed studies demonstrated that the depressions in reproductive hormones were associated with multiple disruptions in the control of reproduction (Van Der Kraak et al. 1992). The negative reproductive responses have not been seen universally, as other studies on feral fish populations downstream of modernized Canadian and Swedish pulp mills found no differences in gonadal development or circulating steroid hormone levels (Kloepper-Sams et al. 1994, Landner et al. 1994, Swanson et al. 1994). However, it is clear that effluent quality varies dramatically within and among mills and that generalizations among sites should not be made.

Alterations in reproductive parameters have been shown to improve after process changes in a variety of sites both in North America and Sweden (Munkittrick et al. 1998). Secondary treatment was installed at Jackfish Bay in 1989, and process changes since 1992 have reduced steroid responses in laboratory exposed fish and in wild fish during early gonadal growth. Secondary sex characteristics in male fish returned to reference situation and both testis and ovarian weight showed significant improvement during the fall period of gonadal growth. However, up to 50 % of female fish still display male secondary sexual sex characteristics, prespawning levels of most hormones have remained at depressed in both sexes, and there has been no recovery of presawning female gonad sizes (Munkittrick et al. 1998, and refs therein). The known process change that took place immediately prior to the partial recovery at the mill site in Jackfish Bay was an increase in bleaching from 50 to 70 % chlorine dioxide substitution. However, increasing chlorine dioxide substitution to 100 %, as well as installation of secondary waste treatment, has not resulted in improvements in circulating steroid hormone levels or gonad sizes in wild fish at Smooth Rock Falls (Munkittrick et al. 1998).

It is not known what process changes are directly responsible for reducing the reproductive responses. Kovacs et al. (1995a, 1996) conducted a series of lifecycle tests, where fathead minnows were exposed to effluent from a bleached kraft mill. Fish exposed to 20 % effluent did not spawn, and fish exposed to 10 % effluent experienced a 116-day delay in maturation. The threshold for spawning and egg production responses was calculated to be 1,7% effluent. A follow-up study conducted after a series of mill upgrades and process changes, revealed no effects on reproductive parameters at concentrations as high as 20 % effluent (Kovacs et al. 1996). This was consistent with field collections as wild spoonhead sculpin collected downstream of this mill's discharge after modernization were larger than upstream fish and showed no decreases in reproductive parameters (Gibbons et al. 1996). Mill process changes implemented between the two studies by Kovacs et al. (1995a, 1996), included increasing ClO_2 substitution from 45 to 100, improved brownstock washing, increased efficiency of the steam strippers and increased recycling of foul condensates, changes in some sewer destinations, and increasing the retention time in the secondary treatment system. It is not known which changes contributed to the improvement in reproductive responses, but it is clear that this and other mills have recently improved the quality of their effluents and in many cases reduced the reproductive consequences (Munkittrick et al. 1998). Although improvements have been detected in physiological parameters in fish after secondary treatment, wild fish movements have not been restricted and the reduction in acute toxicity of the effluent after secondary treatment could result in fish increasing their exposure to effluent. Secondary treatment is effective at reducing chemical oxygen demand, suspended solids, and the temperature of the effluent discharges, all of which would allow fish to inhabit higher effluent concentrations. Studies from Jackfish Bay suggest that the compounds responsible for changes in reproductive parameters are present in secondary-treated bleached kraft mill effluents. The responses seen in wild fish did not correlate with bleaching process, the presence of secondary treatment, or use of chlorine (Munkittrick et al. 1998).

The chemicals responsible for the reproductive effects in fish downstream of pulp and paper mills are not known. It seems unlikely that persistent bioaccumulative compounds such as the more chlorinated dioxins or furans are responsible for the altered reproductive responses. All bleached kraft pulp mills in Scandinavia and a large number in North America have eliminated dioxins and furans from effluents through various process upgrading measures. These include introduction of chlorine dioxide bleaching or totally chlorine-free bleaching, efficient chemical mixing, and secondary effluent treatment, but these technology improvements did not completely eliminate effluent effects on mixed function oxygenase (MFO) induction and endocrine function in fish. In addition, fish exposed to effluent from mills that do not use chlorine bleaching have induced hepatic activity and reduced sex steroid levels (Munkittrick et al. 1994, McMaster et al. 1996a). Sampling 10 days following a mill maintenance shutdown revealed recovery of biochemical responses to BKME as the level of MFO induction was reduced and plasma sex steroid levels had increased to levels near those found in reference fish. This pattern of response is unlike that observed with persistent bioaccumulative organochlorines and suggests that chemicals responsible for the reproductive responses were relatively short-lived and that continuous exposure is needed for the endocrine responses to persist (Van Der Kraak et al. 1998b).

6.3 Reproductive responses observed in laboratory experiments with pulp and paper mill effluents

Laboratory tests using fathead minnows (*Pimephales promelas*) exposed over their life cycle to BKME have confirmed the depression in sex steroid production, the delay in sexual maturity, reduced egg production and changes in secondary sexual characteristics (Robinson et al. 1994). However, responses were only seen at concentrations above those seen in receiving waters at that site. As already mentioned in chapter 5.1 separate studies on fathead minnows exposed to a different BKME showed impacts on time of maturation, egg production and gender (Kovacs et al. 1995a,) with a threshold for spawning and egg production calculated to be 1.7 %. These studies provided evidence that the reproductive responses are directly associated with effluent exposure and are not simply the result of habitat alterations.

In an experiment where two pike populations, one from a clean are and one from a discharge area, were compared, a very significant difference was obtained (Tana & Nikunen 1986). The eggs of pikes caught from the discharge site of a pulp and paper mill were more resistant to the effluent than the eggs of fish from clean area; a higher percent of eggs stayed alive in all effluent concentrations (0.5 %, 2.5 %, 10 %) used. Quite a different result was obtained when some of the eggs from both parent groups were transported to an other laboratory after one week of incubation. Hauling did not affect the viability frequency of the reference group but the additional stress sharply increased the mortality of the exposed population. Accordingly, the pike population that lived in the recipient site showed indices of acclimatization to strongly altered water quality. The difference between pike populations was especially large when the impact of BKME at the fertilisation moment was studied. Hauling, however, strongly increased the mortality of the eggs of the exposed pike population. In this case the intolerance to hauling may indicate that the acclimatization to pulp and paper effluent had diminished the capacity to survive in simultaneous stress. Consequently, the advantage of the adaptation may in practice be questionable (Tana & Nikunen 1986).

In a series of recent studies, sexually immature rainbow trout were exposed for 21 d to the effluents from two Canadian pulp mills producing bleached pulp (mills PM1 and PM2) (Tremblay & Van Der Kraak 1999). PM1 is a bleached sulphite mill using 60 % chlorine dioxide substitution, and having a wood furnish consisting of both softwood and hardwood. Mill PM2 is a softwood bleached kraft mill using 100 % chlorine dioxide substitution. Both mills have biological effluent treatment. Effluent concentrations tested were 10, 35 and 70 % effluent. Plasma VTG levels were significantly elevated in all exposures, although the only exposure producing substantial elevation in VTG levels was the 70 % effluent from PM1. No clear reduction in plasma testosterone levels or in plasma cholesterol levels was observed in the exposed fish, but both effluents caused significant induction of the hepatic EROD activity.

In experimental life-cycle studies, where fish were exposed to secondary treated effluent from TMP mills, these effluents (up to concentrations to 20 %) did not induce any changes in growth or reproduction-related endpoints such as spawning delay, egg hatch or altered sex ratios of the fish (Kovacs et al. 1995b). However, exposure for 4 months of sexually mature brown trout to diluted, untreated effluent (0.13 % concentration) from a peroxide- bleached TMP mill caused lower hatching success than control fish, while pre-exposure to treated effluent did not influence egg hatchability. Moreover newly hatched larvae descending from pre-exposed parents generally exhibited a slower growth rate than larvae from control parents. Since this impact on reproduction was accompanied by a clear depression of the plasma testosterone level in the exposed fish, it was suggested that the effects were mediated by some endocrine modulating substances in the effluents (Johnsen et al. 2000).

There are differing opinions about the choice of appropriate test concentrations for laboratory exposures, and the choice must be affected by the purpose of the study. Some investigators choose a maximal concentration equivalent to, or close to, the maximum observable concentration in the receiving environment for that pulp-mill. The selection method may be relevant if the purpose of the study is to determine whether there are likely receiving water impacts, especially if the receiving environment is unstable at that site. Other studies may be aimed at determining whether a specific process results in the production or release of compounds of concern. In this case, exposure concentrations should be maximized, especially given the apparent differences in sensitivities observed between field and laboratory exposures (Munkittrick et al. 1998).

7 Hormonal effects of municipal sewage effluents

Some of the most compelling evidence for effects of environmental EDs comes from studies examining fish downstream of sewage treatment works in United Kingdom. An extensive series of studies using caged fish showed that exposure to effluents was associated with massive increase in plasma vitellogenin levels. Elevation of vitellogeninsynthesis is diagnostic of exposure to estrogenic compounds. Subsequent studies suggest that natural estrogens, synthetic estrogens from birth control pills and chemicals with estrogenic activity (alkylphenols) present in the effluent may contribute to these responses. At this time, evidence of a linkage between exposure to estrogenic substances and adverse population level outcomes in fish is much weaker. However, there is evidence that there is decreased testicular growth in caged rainbow trout concomitant with vitellogenin induction. Recent observations of elevated vitellogenin levels in fish at selected sites in North America raise the possibility that risk posed by sewage treatment effluents may be widespread (Van Der Kraak 1998).

In the first instance estrogenic effects of sewage treatment works (STW) were detected by measuring VTG in the blood of male fish. Using caged rainbow trout, Harries et al. (1997) have shown that some UK rivers downstream of STWs discharges are estrogenic for several kilometers, although the effect usually rapidly declines with distance due to dilution and other processes. While undiluted STW effluent caused up to one-million-fold induction of plasma VTG in rainbow trout, exposure to 25 % STW effluent did not result in any VTG induction. Male fish collected from effluent channel below the St. Paul metropolitan sewage treatment plant had significantly elevated serum VTG concentrations and significantly decreased serum testosterone concentrations compared to male carp from the St. Croix River, classified as National Wild and Scenic River. Carp collected from the Minnesota River, which receives significant agricultural runoff, also exhibited depressed serum testosterone concentrations, but no serum vitellogenin was apparent (Folmar et al. 1996).

Although VTG production in males (and immature females) might not necessarily imply that reproductive function is impaired, the prolonged synthesis in males of what are in some cases extremely high VTG concentrations could cause serious metabolic stress, leading to kidney and liver damage and necrosis (Herman & Kincaid 1988), diversion of vital amino acids/lipids, and loss of calcium from the scales and skeleton (Carragher & Sumpter 1991), perhaps increasing susceptibility to disease and possibly death (Allen et al. 1999).

In experimental life-cycle studies, where fish were exposed to effluents from sewage treatment works, altered sex ratios were observed in first and second generations. In these experiments, parental zebrafish (*Brachydanio rerio*) were exposed to 20 % (v/v) effluents. In the first generation that majority of fry were males, whereas in the second generation more than 80 % of the fry were females. In several males of the second generation female gametes were also found in quite extensive amounts. The altered sex ratios were even seen in fry, which were allowed to swim in clear water. It is possible that the disruption is transformed to fry via DNA from the parental fish (Tardier Ankara, personal communication).

Retardation of testicular growth has been documented in caged rainbow trout exposed to STEW effluents, and this effect has been replicated in laboratory experiments with alkylphenols (Jobling et al. 1996). Recent results have shown that this estrogenic exposure is accompanied in most instances by the presence of ovotestis (oocytes being present in testes) or intersex conditions in wild roach (Jobling et al. 1998). In some rivers, 100 % of the male roach exhibited this intersex condition, and effects are more marked below STW discharges in comparison with upstream stretches. In UK no roach populations appear to be totally free of ovotestis, but it is not known whether background levels of this condition are natural or due to the absence of completely pristine surface waters (Matthiessen et al. 1998). Recently, the same kind of intersex conditions as had been observed in roach from various UK rivers was found for the first time in a wild marine species caught in a UK estuary. In 1996, almost 20 % of the male flounders from the Mersey estuary contained oocytes in their testes, but the intersex condition was not seen in the three other estuaries investigated (Allen et al. 1999). Unexpectedly, in view of the high levels of VTG induction, no intersex fish were seen in the Tees estuary, but this was probably due to the small sample size from this estuary. Broadly speaking, ovotestis only seemed to occur in flounder populations when mean plasma VTG levels in male adults exceeded 100 µg/ml (Matthiessen et al. 1998).

The first study to investigate the presence of estrogenicity in the males of wild marine fish species was by Lye et al. (1997), who observed that significant proportion (60 %) of male flounder (*Platichtys flesus*) in the Tyne estuary contained VTG and that average concentrations were higher than those found in mature females from the control estuary, Solway Firth. Malformed testes were not observed in any fish from the Solway Firth. All males from this estuary were found in spawning/spent condition. However, in the Tyne estuary, malformed testes were found in a substantial proportion (30-35 %) of the examined male flounders from the sites surveyed, and readily running milt was absent in the majority of males from Tyne.

Later surveys, during the period 1996 to 1998, of plasma VTG levels in male flounder from 11 UK estuaries clearly showed that these were significantly elevated (in comparison with clean reference site on the Alde estuary) in at least one sample from the most of the 11 estuaries. VTG concentrations in Tees, Mersey and Tyne male flounders were extremely high (>100 µg/ml), and often exceeded those normally found in sexually mature females (Matthiessen et al. 1998).

Ovotestis is almost certainly induced though exposure of flounder larvae at the stage of gonadal development, and not through exposure of juveniles or adults. Supporting evidence for this is that intersex adults show no consistent pattern of VTG induction, suggesting that their condition was induced much earlier in their life history. The precise age of gonadal development in flounder is unknown, but it seem likely that it occurs while majority of larvae are still at sea. This would tend to minimise the appearance of intersex in this species, but implies that other species which breed in estuaries are likely to show a higher prevalence of this condition (Matthiessen et al. 1998).

The occurrence of certain natural and synthetic steroidal estogens in the final effluent from STW have been demonstrated (Desbrow et al. 1998). In this study three sterols were isolated from estrogenic fractions of sewage extracts and these were the natural hormones 17-β-estradiol and estrone and the synthetic hormone 17-alpha-ethynylestradiol. 17-β-estradiol and estrone were present in all effluents at measured concentrations ranging from 1 ng/l to almost 50 and 80 ng/l, respectively. The concentration of 17-alpha-ethynylestradiol was generally below the limit of detection, but was positively identified in three of the effluent samples at concentrations ranging from 0.2 to 7 ng/l. These data suggested that natural and synthetic hormones may be responsible for the observed induction of vitellogenin synthesis in male fish placed downstream of effluent discharges from STWs that mainly receive domestic inputs (Desbrow et al. 1998). Subsequent **in vivo** tank experiments, in which rainbow trout and roach were exposed for 21 days via water to environmentally relevant concentrations of 17-β-estradiol, estrone and alkylphenolic xenoestrogen, 4-tert-octylphenol, indicated that natural steroidal estrogens are sufficient to account for the levels of vitellogenin synthesis observed in caged male fish placed downstream of certain STW effluents in British rivers (Routledge et al. 1998).

It is currently not known exactly what are the causative substances for the wide-spread estrogenic effects observed in UK waters. However, alkylphenol ethoxylates and their degradation products used in industrial cleaning and wool processing plants, as well as the natural endogenous estrogens 17-β-estradiol and estrone, and pharmaceutical estrogens, such as ethynyl estradiol, have all been implicated in the anomalies observed in feral or caged male fish below STWs in UK (Purdom et al. 1994). Later it has been concluded that it seems unlikely that the major causative substances will prove to be estrogenic hormones derived from domestic sewage, although these materials are almost certainly contributing to the observed effects. There is little correlation between VTG induction and the volumes of domestic sewage discharges to each estuary, but there is a much clearer relationship with the volume of industrial effluent (Matthiessen et al. 1998). However, this does not prove a causal link with industrial chemicals, but it suggests that non-hormonal substances are major players.

Ecological significance of endocrine disruption

8

An ecological risk assessment of the potential effects of EDs in feral fish should pay particular attention to the fundamental factors that dictate ecological sustainability. In this context, a paradigm of evolutionary biology is that, under optimal environmental conditions, there is a reproductive surplus in a population and that the consequent competition between individuals drives the process of natural selection (Maynard Smith 1993). Natural selection therefore nurtures the individuals with greatest fitness, namely the individuals with the ability to reproduce and survive. Protection of these essential parameters should be the fundamental goal of environmental (ecological) risk assessment for EDs and other potential chemical contaminants (Campbell & Hutchinson 1998).

The endocrine system is involved with chemical communication and serves to regulate and co-ordinate diverse physiological processes including reproduction, growth, maintenance of the internal environment and energy availability. Hormones serve as the chemical messenger and, as is the case for their effects on reproduction, play both an organizational role defining the direction and functional extent of development and an activational role in stimulating changes in the various components of the system. Therefore there is a broad range of effects that may be mediated by environmental chemicals that influence the endocrine system. This may encompass effects which influence the functional integrity of the exposed adults as well as having an impact on development of the offspring. Understanding the potential actions of EDs in wildlife is further complicated by our limited understanding of the basic endocrinology and physiology of all but a few species. Scientists are also dealing with species which use a diversity of reproductive strategies (oviparity, ovoviviparity, viviparity, delayed implantation) and often have unique developmental characteristics (e.g. smoltification, metamorphosis) which generally have unknown sensitivities to EDs (Van Der Kraak 1998).

There has been an increased reliance on the use of physiological endpoints to evaluate the effects of suspected EDs in wildlife. Cellular and molecular measures such as receptor binding and gene activation tests have been instrumental as screening methods for suspected EDs and in terms of defining their mechanisms of action. Similarly, physiological endpoints such as the measurement of plasma vitellogenin and sex steroid hormone levels are now commonplace in non-mammalian species. However, caution is warranted when interpreting these endpoints owing to the uncertainty of whether the biochemical and physiological responses are related to changes in whole organism performance in terms of effects on growth, reproduction or survival. For example, even though endocrine signalling is necessary for proper reproductive functioning, the identification of hormonal alterations does not necessarily equate with impairment of reproductive success. The impact of such a change on an organism will depend on a number of factors including the hormone affected, degree of impairment, timing of the change and the relative species sensitivity (Van Der Kraak 1998).

An overriding concern with all ecotoxicological investigations is that a variety of factors in addition to endocrine disruption can adversely impact growth, reproduction and survival. Quite often these are ignored when assessing wildlife populations for the effects of suspected EDs. Food availability, disease state, competition and loss of habitat are significant stressors to wildlife, yet our understanding of how these contribute to physiological fitness is often inadequate. The diverse environmental factors include also xeno-estrogens and other chemicals observed to affect the reproductive physiology of aquatic organisms. It is also recognised that environmental stress can affect survival and immunocompetence of aquatic organisms and that such stress can also impair the reproductive success of the species. Therefore, there is no doubt that a variety of factors impinge upon the reproductive output and fitness of natural populations. To ensure that appropriate actions are taken to protect the environment, the debate on endocrine disruption in wildlife needs to broaden and to take further account of the ecological dimensions (Campbell & Hutchinson 1998).

It has been repeatedly shown in the laboratory that endocrine disrupting compounds, with artificial or natural origin, have the potential to perturb sensitive hormone pathways that regulate reproductive functions. In fish, this may result in reduced gonad size and decreased egg production in females. It is also known that male fish exposed to estrogenic compounds show induced production of vitellogenin, but the organismal significance of elevated vitellogenin levels has only been, for the most part, speculative (Arcand-Hoy & Benson 1998). Considerable discussion and debate has also occurred concerning the concentration of endocrine disrupting compounds and whether they are sufficiently elevated to exert whole animal effects. With the exception of a few well-documented findings such developmental effects in wildlife associated with exposure to polyhalogenated aromatic compounds, the ecological implications of organismal exposure to endocrine disrupting compounds have, in general, not been established (Arcand-Hoy & Benson 1998).

A review of Canadian field studies on the potential of pulp and paper effluents to affect the reproductive capacity of fish (Kovacs et al. 1997) indicated that the findings and conclusions of the work conducted at different locations were conflicting. This is perhaps not surprising in view of the variety of different approaches utilized, quality of effluents discharged, types of recipients and species studied as well as the confounding effects due to habitat and natural variability. Undoubtedly, in some studies, there were differences in the reproductive parameters (e.g. gonad size, blood steroid levels) for fish collected from reference and effluent exposed sites. In other studies this was not the case. However, even when differences were noted in reproductive indicators, the ecological relevance of such differences at the population and community level was poorly understood. Furthermore, because natural and anthropogenic sources of stress can elicit similar responses, it is difficult to clearly discern if the reported differences were exclusively related to effluent exposure or to other factors such as habitat and natural variability. The interpretation of field studies can also be hampered by inadequate sample size and absence of a clear response which is proportional to effluent concentrations (Kovacs et al. 1997).

It has been concluded from field studies both in North America and Scandinavia that the successive upgrading of the environmental standard of pulp mills has resulted in improvements of the reproductive success and recruitment of feral fish in the near-field receiving waters of these mills. Among the well-studied cases, the most complete recovery of the fish communities in pulp mill effluent receiving waters have occurred in the vicinity of bleached kraft mills, which have concentrated on in-plant measures such as extended cooking, oxygen delignification, advanced black-liquor spill collection system and optimization of process steering. Less evident improvements have been detected outside mills which have given priority or secondary (biological) treatment of their effluents without taking simultaneous in-plant pollution-abating measures. Although it has not been conclusively demonstrated, it may be speculated that the possible explanation for this is that containment and destruction of substances dissolved from the wood, including the wood extractives, in the production process may be a more effective means of obtaining sufficiently low concentrations of active EDs in the receiving waters to avoid biological effects than reducing their concentrations in the effluents by means of secondary treatment facility. Such strategy will also minimise the risk of formation of biologically active metabolites (e.g. retene) in the effluent treatment plan.

9 Conclusions and research needs

Finnish Environment Institute (FEI) Chemical Division, in partnership with the Finnish Institute Occupational Health, has conducted a project on environmental risks management of endocrine disruptors. Finnish Environmental Research Group has produced a review of the available knowledge of endocrine disrupting natural compounds and endocrine effects of pulp and paper mill and municipal sewage effluents as part of this project. For further clarification of the objective of the review, it should be stated that it serves as a background paper and not a programme for organised research. The review does not give a thorough assessment of risks but is a general analysis attempting to identify some of the relevant principle questions and approaches to answer them.

The review gives a general background of plant derived natural substances with hormonal effects and a more detailed description of known hormonally active phytoestrogens and their sources. A short description of the endocrine system in fish and different screening-tests for detecting endocrinologically active compounds is also included in the review. Finally the available knowledge of hormonal effects of effluents from pulp and paper industry and sewage treatment works is presented and the ecological significance of endocrine disruption discussed. In this short summary the concluding remarks of the review and further research areas based on those conclusions are presented.

9.1 Conclusions

Ether extractable constituents of spruce and pine wood (so-called ″wood extractives″) make up 1-2 % (spruce) and about 4 % (pine) of total wood mass. Each of at least four groups of chemical compounds, belonging to wood extractives, i.e. the lignans, the stilbens, the resin acids and the sterols/triterpene alcohols, contain individual components that have been demonstrated to exert endocrine activity in relevant test systems. Some of these components show endocrine activity only after microbial transformation, for example, the resin acid metabolite retene, formed in anaerobic environments, such as anoxic bottom sediments.

Wood extractives are regularly detected in the effluents of kraft and thermomechanical pulp production, the best investigated groups with potential endocrine activity being resin acids and the sterols. Concentrations of total resin acids in the final effluents from modern pulp mills range between 830 and 1 600 µg/l before treatment and between 3 and 12 µg/l after activated sludge treatment. In the case of phytosterols and triterpene alcohols, in modern Scandinavian kraft pulp mills, total concentrations in untreated effluents range from 280 to 3 000 µg/l, while those in activated sludge-treated effluents were 20-50 µg/l. Untreated effluents from thermomechanical pulp production contain in general much higher levels of wood extractives than untreated effluents from production of chemical pulp and the same trend seems to be also with treated effluents.

Attempts to identify the compounds in pulp mill effluents that are responsible for the observed endocrine modulating effects in aquatic organisms have not yet been entirely successful. However, it has been demonstrated that at least one plant sterol, occurring in the wood raw material - and in the effluents - i.e. β-sitosterol, is capable of inducing series of estrogenic responses in fish, with a treshold effect concentration in water close to 10 µg/l. Moreover, the resin acid metabolite, retene, causes a variety of lesions in fish embryos and larvae at concentrations in the range of 16-32 µg/l. Nevertheless, it is clearly indicated that, in addition to β-sitosterol and retene, there are other compounds present in mill effluents and in the discharge receiving waters that affect reproductive endpoints in aquatic organisms e.g. compounds acting as anti-estrogenic or androgen agonists.

The studies performed in several receiving waters have shown that effluents from pulp and paper mills that meet the regulations governing dioxins, oxygen demand, nutrients and acute lethality can be associated with subtle changes in the reproduction processes of wild fish. These reproductive changes, including delays in reaching sexual maturity and decreases in fecundity, have been duplicated in long-term and short-term laboratory exposures to some pulp mill effluents. The changes in reproductive parameters appear to be caused by disruptions in the hormonal systems regulating reproduction at a number of sites within the pathways.

Study design, habitat characteristics, species habitat preferences and fish mobility play important unidentified roles in contributing to the degree of impact evident at some sites. In cases where receiving water responses have not been observed in wild fish, it is unclear if the absence of responses is related to a lack of production of compounds capable of impacting reproduction, or whether the receiving environment at those sites protect wild fish from the biochemical changes. It is also clear that effluent quality varies dramatically within and among mills and that generalizations among sites should not be made.

Improvements have been seen at several sites over the past few years, and it now appears that pulp and paper mills can make process changes that significantly reduce their impact on reproduction in aquatic organisms. However, it is not known what process changes are directly responsible for reducing the reproductive responses. It should also be stressed that most of the studies have been made in North-America outside old-fashioned mills and very few studies outside modern mills from Scandinavia. As a consequence generalizations of effects observed in North-America may not be true for mills in Scandinavia. The responses in wild fish have not been seen to correlate with bleaching process, the presence of secondary treatment, or use of chlorine.

While extensive evidence have been provided that pulp mill effluents modulate various aspects of reproductive function in aquatic organisms, it is not easy to establish cause-and-effect linkages for either individual chemicals or groups of chemicals acting through a common mechanism. Part of the complexity in evaluating the mechanism of action of individual compounds or complex mixtures comes from the need to make extrapolations from feral fish populations to laboratory exposures. The differences between test species in the laboratory and field settings, variation in the duration of exposure, and changes in responsiveness with developmental status complicate interpretation of responses and add to the difficulties in establishing linkages. It is also noteworthy that there is hardly any information of other industrial effluents than pulp and paper.

Some of the most compelling evidence for effects of environmental EDs comes from studies examining fish downstream of sewage treatment works in United Kingdom. An extensive series of studies using caged fish have shown that exposure to effluents was associated with massive increase in plasma vitellogenin level and substantial increases in endocrine responses have been recorded in feral fish. It is currently not known exactly what are the causative substances for the widespread estrogenic effects observed in receiving waters of sewage treatment works, but subsequent studies suggest that natural estrogens, synthetic estrogens and chemicals with estrogenic activity (e.g. alkylphenols) present in the effluents may contribute to these responses.

The ecological significance of endocrine disruption in aquatic organisms has been debated for a period of time. The precise endocrine mechanisms that influence the reproductive success of a species are diverse and are affected by a range of physicochemical and biological factors. In addition to natural or man-made endocrine-disrupting compounds, it is recognised that environmental stress can affect the survival and immunocompetence of fish populations and that such stress can also impair the reproductive success of the species. Moreover, nutritional deficiencies may have profound impacts on the survival, development and reproductive health of aquatic organisms. Therefore, there is no doubt that a variety of factors impinge upon reproductive output and fitness of natural populations. In relation to the endocrine action of some components (and/or metabolites) of the wood extractives occurring in the effluents of pulp and paper mills, it should be kept in mind that these compounds are natural constituents of plants that have always occurred in the environment. Thus, aquatic organisms have always been exposed to low concentrations of these compounds and may have developed homeostatic mechanisms to cope with them.

Effects of endocrine disrupters on other organisms than fish are lacking. This must be considered as a weakness since reproductory effects on fish may not reflect effects on classes of other organisms. Concequently effects on key ecosystem mechanisms may not be detected. Short-term toxicity tests have been conducted with a variety of aquatic invertebrates, but with a few exceptions, the most common chronic reproduction tests used for regulatory purposes are with parthenogenic stages of various daphnid species. This, of course, limits the ability to detect compounds that might affect sexual reproduction (Ankley et al. 1998).

9.2 Research needs

Based on the conclusions from the present review, following areas of further research are given below.

1) Mill process related chemical/biological processes
2) Experimental work on reproductory processes in fish and other aquatic organisms and their seasonal dependence
3) Based on results from point 2, directed research on key-mechanisms in feral fish populations and quantification of the possible effect and its reflection on ecosystem structure and function.

In item 1 chemical analytical work needs to be done in order to verify if endocrinologically active compounds occur in pulp and paper and sewage treatment work effluents, and if such compounds are explicitly formed during the treatment processes. The amounts formed need to be quantified preferably from all major effluent contributors in order to obtain a preliminary knowledge on the load on a whole country basis.

Item 2 includes further experimental research on endocrinologically relevant mechanisms in fish during the reproductory cycle, including long-term exposures to pulp mill and sewage treatment work effluents. It is recommended that exposures are performed using both untreated and treated effluents. Organic carbon should be used as dilution factor in order to establish differences in biological activity of organic carbon before and after treatment.

Basic knowledge is lacking regarding annual endocrinological cycles in feral fish species (**item 3**) as well as ecological significance of specific feral fish species. Consequently before biomarkers for detection of subindividual effects are developed, a better understanding on the significance of natural factors on energetics, growth and reproduction should be obtained. This would increase the knowledge how relevant subindividual biomarkers reveal cause-effect relationships at population level.

In several publications and reports the need for new short-term tests have been stressed. Based on the present review it is rather suggested that additional work is required on the correct application of the already existing methods. In particular it is recommended that future research efforts focus on an integrated approach using a combination of field work, mesocosm experiments and laboratory tests. This is especially true for studies on the impact of pulp and paper mill discharges and sewage treatment work effluents due to the complexity of effluents and site-specific differences in both effluent and receiving water quality.

Finland, being part of the boreal region, is taking a special position with countries of the same latitude regarding reproduction of aquatic organisms. This is due to the fact that the ″window″ for reproduction is short and restricted to a limited time interval. Any disturbances before or during these periods may lead to reproductory failure and research efforts should be directed towards these sensitive periods.

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Documentation page

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Author(s)	Karl-Johan Lehtinen and Jukka Tana	
Title of publication	Review of endocrine disrupting natural compounds and endocrine effects of pulp and paper mill and municipal sewage effluents (Teollisuus- ja yhdyskantajätevesien sekä luonnonperäisten aineiden hormonaaliset vaikutukset - kirjallisuuskatsaus)	
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Abstract	The objective of the present report is to give a review of the available knowledge of endocrine disrupting natural compounds and endocrine effects of pulp and paper mill and municipal sewage effluents. The review does not give a thorough assessment of risks but is a general analysis attempting to identify some of the relevant principal questions and approaches to answer them. While extensive evidence have been provided that pulp mill and municipal sewage effluents modulate in various aspects of reproductive function in aquatic organisms, it is not easy to establish cause-effect linkages for either individual chemicals or groups of cemicals acting through a common mechanism. Part of the complexity in avaluating the mechanism of action of individual compounds or complex mixtures comes from the need to make extrapolations from feral fish populations to laboratory exposures. The differences between test species in the laboratory and field settings, variation in the duration of exposure, and changes in responsiveness with developmental status complicate interpretation of responses and add to the difficulties in establishing linkages. It is also noteworthy that there is hardly any information of other industrial effluents than pulp and paper.	
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Tekijä(t)	Karl-Johan Lehtinen ja Jukka Tana	
Julkaisun nimi	Teollisuus- ja yhdyskantajätevesien sekä luonnonperäisten aineiden hormonaaliset vaikutukset - kirjallisuuskatsaus (Review of endocrine disrupting natural compounds and endocrine effects of pulp and paper mill and municipal sewage effluents)	
Julkaisun osat/ muut saman projektin tuottamat julkaisut	Julkaisu on saatavana myös internetistä: http://www.vyh.fi/eng/orginfo/publica/electro/fe447/fe447.htm	
Tiivistelmä	Tämän kirjallisuusselvityksen tavoitteena on ollut laatia olemassa olevaan aineistoon perustuva katsaus luonnoperäisten yhdisteiden sekä teollisuus- ja yhdyskuntajätevesien hormonaalisista vaikutuksista. Tämä katsaus ei sisällä perusteellista riskiarviota vaan pyrkii antamaan yleisluonteisen analyysin aiheeseen liittyvistä kysymyksistä ja tutkimustarpeista. Vaikka metsäteollisuuden ja yhdyskuntajätevesien vaikutuksista vesieliöiden lisääntymiseen on olemassa paljon havaintoja ovat yksittäisten kemikaalien tai kemikaaliryhmien aiheuttamat syy-seuraussuhteet eliöiden lisääntymistoimintoihin edelleen tuntemattomia. Yksittäisten yhdisteiden tai jätevesien vaikutusmekanismien arviointia ovat osaltaan vaikeuttamassa laboratorioaltistuksissa saatujen tulosten soveltaminen luonnon olosuhteisiin. Tutkimuksia on tehty monilla eri lajeilla ja kehitysasteeltaan erilaisilla yksilöillä, jotka yhdessä kestoltaan erilaisten altistusaikojen kanssa mutkistavat tulosten tulkintaa. Huomionarvoista on myös se, että metsäteollisuutta lukuunottamatta muiden teollisuusjätevesien hormonaalisista vaikutuksista on hyvin vähän tietoa.	
Asiasanat	hormonit, biologiset vaikutukset, luonnonyhdisteet, jätevedet, metsäteollisuus, kunnat	
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Presentationsblad

Utgivare	Finlands miljöcentral	Datum Januari 2001
Författare	Karl-Johan Lehtinen och Jukka Tana	
Publikationens titel	Litteraturstudie avseende naturliga endokrina substanser samt effekter av skogsindustriella och kommunala avloppsvatten (Review of endocrine disrupting natural compounds and endocrine effects of pulp and paper mill and municipal sewage effluents)	
Publikationens delar/ andra publikationer inom samma projekt	Publikationen finns tillgänglig på internet: http://www.vyh.fi/eng/orginfo/publica/electro/fe447/fe447.htm	
Sammandrag	Syftet med föreliggande rapport är att ge en översikt av tillgänglig information om naturligt förekommande endokrint störande substanser samt endokrina effekter av skogsindustriella och kommunala utsläpp. Översikten omfattar inte en grundlig riskvärdering, snarare har strävan varit att göra en generell analys med sytftet att identifiera vissa relevanta principfrågor samt sättet att besvara dessa. Trots att ett stort antal bevis föreligger på att skogsindustriella och kommunala utsläpp påverkar förökningsmekanismer hos vattenorganismer, är det inte lätt att etablera ett orsak-verkan förhållande för vare sig enskilda kemikalier eller grupper av kemikalier som utövar sin verkan via en gemansam mekanismer. Komplexiteten i utvärderingen beror delvis på svårigheter vid extrapoleringen mellan fältresultat och resultat erhållna vid laboratorieförsök. Skillnaderna mellan testorganismerna i laboratorium och fälltsituationen, variationen i exponeringstid och förändringarna i organismernas känslighet beroende på utvecklingsstadium komplicerar resultattolkningen och bidrar till svårigheterna att etablera orsakssamband. Värt att notera är även att annan information om industriella avloppsvatten än skogsindustriella nästan helt saknas.	
Nyckelord	hormoner, biologiska effekter, naturliga substanaser, skogsindustri, kommuner, avloppsvatten	
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ENVIRONMENTAL PROTECTION

Review of endocrine disrupting natural compounds and endocrine effects of pulpand paper mill and municipal sewage effluents

In recent years the term ”endocrine disruptors” has become increasingly common in the vocabulary of professional ecotoxicologists and in the media. The meaning of this term is that substances after entering the body of an organism interacts with the organism’s hormonal system usually, in some way or the other, by interfering with normal hormonal processes. The occurence of exogenous chemical substances with endocrinological effects has raised concerns that anthropogenic substances may cause widespread effects in both humans as well as in wildlife.

The objective of this report is to give a review of the available knowledge of endocrine disrupting natural compounds and endocrine effects of pulp and paper and municipal sewage effluents. The present review is an attempt to give a general analysis based on current knowledge and to identify some of the relevant principal questions and approaches to answer them.

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