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Feminisation of fish

The effect of estrogenic compounds and their fate in sewage treatment plants and nature

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Preface

This report aims to give an overview of the existing knowledge on selected endocrine disrupting compounds including their fate in sewage treatment works, their occurrence in the aquatic environment and their endocrine disrupting effects on fish. Both effects observed among wild species of fish in their natural environments and effects which can be induced by controlled exposure of fish to the compounds in question will be described.

Studies from a number of European countries during recent years have demonstrated that feminisation of fish in their aquatic environment in many cases can be ascribed to the natural steroids, 17β-estradiol and estrone, and the synthetic estrogen, ethinylestradiol, used in contraceptives and all being excreted with domestic sewage by women. The main focus of this report will therefore be on these three hormones. Additional information will be given in lesser detail on the less potent natural estrogen estriol and on alkylphenols and bisphenol A which are known estrogenic chemicals which can be detected in sewage effluent. This report will therefore concentrate on endocrine disruption via estrogenic mechanisms, though it should be born in mind that endocrine disruption is a larger subject which also covers i.e. antiestrogenic, androgenic and antiandrogenic effects. The latter are, however, outside the scope of this report.

1 Summary (English)

Feminisation of male fish in freshwater and marine environments

During the past ten years feminisation of male fish has been detected in several European countries and in USA and Japan (*1-12*). These cases are examples of disturbances of the male reproductive system which are believed to be consequences of endocrine disruption caused by female hormones, estrogens, or chemicals mimicking estrogens present in the aquatic environment. The majority of estrogens are believed to reach the aquatic environment by sewage effluent. In Europe feminisation of male fish exposed to sewage effluent has been seen in England (*1*), Sweden (*3*), Norway (*4*), Germany (*5*), The Netherlands (*6*), France (*7;13*), Spain (*8;14*) and Denmark (*2*). Further, the observations of feminisation in wild populations of fish have been made in a range of different species including the freshwater species roach (*1*), gudgeon (*15*), carp (*8;14*), bream (*5;6*), chub (*7;13*) and brown trout (*2*) and the saltwater species flounder(*16-22*), eelpout (*18*) and two species of sand gobies (*23*). Thus, these kinds of disruption of the male reproductive system have both been found in freshwater and marine environments though most cases have been reported from freshwater environments. Controlled exposure of fish to sewage effluent i.e. in cage experiments have further added proof to the estrogenic nature of the sewage effluent and estrogens or estrogenic chemicals as causative agents of the disruptions.

The signs of feminisation in male fish are the production of a female yolk protein which is known only to be produced as a response to an estrogen exposure, and the occurrence of intersex – an abnormal form of hermaphroditism. Males with the intersex condition have early stages of egg cells in the testis and in some cases they have also developed the female duct which leads eggs to the oviduct. Feminisation has been found in varying degrees among individual fish from mild to very severe disturbances of the male reproductive system.

England is the country in which feminisation of fish has been most widely detected and where the most severe degrees of feminisation have been seen, both compared to countries inside and outside Europe. The feminisation of fish which has been detected in Denmark does not, however, differ markedly from the extent found in other countries. In these a lower occurrence and severity of feminisation compared to England have generally been seen though feminisation has been detected at several of the examined sites. There are, however, also sites where the fish populations appear unaffected.

It should be pointed out that short-term exposure experiments, where fish are exposed to sewage effluent for a short period of time, do not always give a correct estimate of the risk for wild populations of fish which live their entire life in the stream or river. It has been demonstrated that increasing the exposure time for sewage effluent lowers the concentration of sewage effluent needed to cause feminisation of fish (*24*).

Estrogenicity of sewage effluent
Support to the theory of estrogenic compounds in sewage effluent as the cause of the feminising effects, which have been found among male fish, has also come from a number of studies using cell culture assays designed to detect estrogenic activity. These have demonstrated the estrogenicity of sewage effluent from a number of countries (England, Germany, The Netherlands, Belgium, China, Korea and USA) and quantified the estrogenic potential in relation to the potency of 17ß-estradiol (*6;25-33*). Further, chemical analysis of the composition of sewage effluent and determinations of concentrations of estrogens and estrogenic compounds in the effluent have in a large number of cases demonstrated the natural estrogens, 17ß-estradiol, estrone and the synthetic estrogen, ethinylestradiol used in contraceptives as likely candidates for the observed disturbances in fish species from the sewage effluent receiving rivers. In single cases alkylphenols were also suggested as possible causative agents (*34*).

Sewage effluent and surface water concentrations of estrogens.
Internationally, the three natural estrogens, 17ß-estradiol, estrone and estriol have been detected in sewage effluent at concentrations of < 0.1 – 88 ng/l, < 0.1 – 220 ng/l and < 0.1 – 42 ng/l, respectively. The even more potent synthetic estrogen, ethinylestradiol has been found at concentrations of < 0.053 – 62 ng/l. The typical range for the steroids are in the range 1-10 ng/l and 5-20 ng/l for 17ß-estradiol and estrone, respectively, while ethinylestradiol often is below detection limits (0.1 – 1 ng/l). When ethinylestradiol is detected it is mostly below 10 ng/l. The concentrations measured in Danish sewage effluent lie within the typical range of estrogen concentrations reported from other Europeans countries. In surface waters, concentrations of estrogens are lower compared to sewage effluent and have been detected at concentrations of 0.05 – 15.5 ng estradiol/l, < 0.1 – 17 ng estrone/l, < 0.1 – 3.4 ng estriol/l and < 0.053 – 30.8 ng ethinylestradiol/l with typical concentrations of less than 5 ng/l for estradiol and estrone and less than 1 ng/l for ethinylestradiol. For both sewage and surface water estrone is the most and ethinylestradiol the least frequently detected estrogen.

An important thing which has to be remembered whenever concentrations of estrogens are mentioned in the present report is, however, that it seldom is specified whether the chemical analysis measures only free or both free and conjugated estrogens. There must probably also be expected to be larger uncertainties related to measurements of estrogen concentrations in influent than in effluent due primarily to interference from large contents of organic matter (matrix effect). Further, the detection limits of the analyses, especially for ethinylestradiol, are close to and sometimes even above the concentrations which have been demonstrated to cause reproductive disturbances in fish.

Fate of estrogens in the aquatic environment
Knowledge on the fate, behaviour and persistence of estrogens in the environment is still relatively limited. The synthetic ethinylestradiol seems, however, to be more persistent than the natural estrogens both in water and sediment. Under aerobic conditions mean half-lives in water of 17ß-estradiol and estrone has been calculated to 2.8 and 3.0 days, respectively (*35*). Ethinylestradiol has been demonstrated to have a half-life ten times that of estradiol under the same incubation conditions (1.2 versus 17 days for estradiol and ethinylestradiol, respectively) (*35;36*). Estrogens have in general a medium sorption potential to sediment (*37*) and low degradation of both

estrone and particularly ethinylestradiol in sediment with low oxygenation might indicate a risk for sediment as a sink for these estrogens (*35*).

Occurrence and fate of selected estrogenic compounds in sewage effluent and surface water

Alkylphenols and bisphenol A are two of the more potent of the estrogenic chemicals which might be released with sewage effluent and which therefore receive some attention in the present report. The most important alkylphenols with regard to estrogenicity are nonylphenol and octylphenol. These are in general detected at concentrations below 10 µg/l in sewage effluent and below 1 µg/l in surface water although examples of concentrations above 300 µg/l in sewage effluent and above 600 µg/l in river water have been reported from some countries. Bisphenol A is less frequently encountered than alkylphenols and has seldom been detected at concentrations above 1 µg/l in either sewage effluent or surface water.

As for estrogens, alkylphenols and bisphenol A will partition between the water phase and the sediment, and indications exist that both have a great potential for accumulating in sediment with anoxic conditions (*38;39*).

The feminising potential of estrogens, alkylphenols and bisphenol A

In light of the observations of reproductive disturbances in fish populations exposed to sewage effluent, a number of controlled exposure experiments have been performed to assess the lowest concentration of estrogens and estrogenic chemicals needed to obtain these observed disturbances. This aids in assessing which compounds might be responsible for the observed effects such as production of yolk protein in males, development of intersex or other disturbances in the male testis.

17ß-estradiol has induced the production of yolk protein at a concentration of 5 ng/l (*40;41*) and has induced intersex at 10 ng/l (*42*). A range of other testicular effects have been seen at concentrations between 10 – 50 ng/l (*43-45*) and also at concentrations above 100 ng/l (*44*). Examples of other effects are inhibition of the normal development of male germ cells, which can be seen as a lower relative weight of the testis and/or a presence of a larger proportion of early stages of germ cells (*44*). The presence of degenerated germ cells has also been seen (*44*).

A similar or slightly lower estrogenic activity has been detected for estrone. Induction of yolk protein and intersex in male fish has been seen at concentrations of 30 (*46;47*) and 10 ng/l (*42*), respectively. Estriol is the least estrogenic of the three natural estradiols. *In vitro* it has been demonstrated to be 30 times less potent than estradiol (*42*) but in regard to induction of intersex *in vivo* it seems to be 100 times less potent (*42*). In general, knowledge on the estrogenic capacity of this estrogen is limited.

Ethinylestradiol is even more potent than the natural estrogens in regard to inducing disruptions in the male reproductive system. Induction of yolk protein and induction of intersex have been seen at 0.1 ng/l (*42*) and changed sex ratio at 0.6 ng/l (*48*). A range of other reproductive effects such as inhibition of normal sperm cell development has been seen at concentrations below 10 ng/l.

Both the alkylphenols, nonylphenol and octylphenol, and bisphenol A have a lower potency than both natural and synthetic estrogens. Effects are seen in the µg/l concentration range. Induction of yolk protein has been detected at 5 µg/l nonylphenol or octylphenol (*49;50*), though at long exposure time with

nonylphenol, the lowest effect concentration was decreased to 1 µg/l (*51;52*). Intersex, changed sex ratio, degenerated testes and inhibited growth of the testes have been seen at nonylphenol concentrations between 30 and 100 µg/l (*50;53;54*). Concentrations down to 2 µg/l octylphenol have caused reproductive disorders in male fish (*49*). Bisphenol A has been demonstrated to exert effects at concentrations between 10 and 40 µg/l (*42;55;56*).

Relationship between effect concentrations of estrogens/estrogenic compounds and their presence in the environment

When comparing the actual sewage effluent and surface water concentrations of the estrogens, alkylphenols and bisphenol A with the lowest concentrations which in controlled laboratory studies can induce reproductive disturbances in male fish, it is seen that concentrations of estradiol, estrone and ethinylestradiol in some cases have been high enough to explain the feminisation of fish. The same holds in some cases for nonylphenol and octylphenol. Reports on environmental concentrations of estriol are too sparse to make a reliable estimation on the possible contribution of this estrogen to feminising effects, while bisphenol A in general has been detected in concentrations below the lowest effect concentration for inducing reproductive disorders in male fish.

In Denmark very few measurements have been performed on water concentrations of estrogens and it is not yet possible to point out the compounds responsible for the observed feminisations of male fish in Danish streams.

When assessing the possible implications for the reproductive health of male fish of the estrogenic compounds in the aquatic environment it is, however, important to remember that the estrogens present in the water will act in an additive manner (*40;57*). Therefore the concentration of a single compound which can exert an effect on males will be lower when present in a mixture of estrogens and xenoestrogens. Further, different fish species show different sensitivities towards endocrine disruption with estrogens, and the timing of the exposure is also important for the resulting effects. The early life stages are generally considered to be the most sensitive stage since the development of the sex takes place at this time (*58*). There might, however, also be certain periods in the reproductive cycle of sexually mature male fish in which they are more susceptible to endocrine disruption.
Intermittent exposure to high concentrations of estrogens have further been demonstrated to result in larger effects than obtained with a continuous exposure to a lower concentration, so release of short pulses of very high concentrations of estrogens and/or estrogenic compounds might have great importance (*59*).

Effect of feminisation or estrogenic exposure on fertility of male and female fish

The observations of feminised fish in many parts of the world have raised the question whether the fertility or reproductive capacity of the fish is reduced and populations in danger of decreasing. Based on the present knowledge this is not yet possible to answer. New results have, however, implicated reduced fertility among intersex roach in England (*57;60*). An asynchrony in the development of germ cells in males and females has been demonstrated due to a delay in the development of male germ cells. Only 50 % of the males were capable of spawning. Further, a reduced milt volume and a reduced density and motility of sperm cells were seen among the other males. This indicates

reduced fertility among the severely intersexed males. The implications of a milder degree of feminisation are harder to estimate.

Controlled exposure studies with estrogens and estrogenic compounds have also demonstrated a reduced fertilisation success as well as altered sexual behaviour of exposed male fish (*49;61-63*).

The impact of production of the female yolk protein in male fish, which is a very widespread phenomenon, on the reproductive capacity is uncertain but could take place possibly via an indirect route. This can be via reduced energy sources to reproduction due to demonstrated effects on liver, kidney and other health conditions (*64-67*).

Most studies both in the field and in the laboratory have focused on the effects of estrogens and estrogenic compounds on the reproductive health of male fish since these are considered to be the most sensitive gender due to their low body concentrations of estrogens. There are, however, also studies which have demonstrated that the reproductive success of females can be reduced by estrogen exposure i.e. via reduced egg spawning. This seems to take place via disturbance of the normal maturation of egg cells (*61;68;69*).

Potential sources of estrogens to sewage

The three natural estrogens are female steroid sex hormones, which are produced naturally, in humans and other vertebrates. Both female and male vertebrates produce estrogens. The production and excretion varies throughout life and between the two sexes. Estradiol is both metabolised reversibly and irreversibly. In the reversible metabolism, estradiol is transformed to estrone, while estradiol is transformed to catechol estrogens or estriol in the irreversible metabolism. The main part of the produced metabolites are finally conjugated with sulphate and glucuronides and excreted in the urine and are as such an important source of natural estrogens in the municipal sewage system. A minor amount is excreted via faeces as unconjungated metabolites (*70;71*).

The production of estrogens in adult men varies in different age groups but the variations are small compared to the variation in mature women. The production and then the excretion of estrogens from the mature women vary during the menstrual cycle and the pregnancy until the menopause is reached. The daily excretion of estrogens in urine by menstruating women is on average: 4.8 µg estriol, 8.0 µg estrone and 3.5 µg estradiol whereas the average daily excretion by pregnant women is: 6,000 µg estriol, 600 µg estrone and 259 µg estradiol (*72*) (*73*). The production of estrogens decline at the menopause and the post menopause production of estrogens is very low (*74*). The average excretion of the three estrogens in urine by postmenopausal women is approx. 7 µg/day. This is at the same level as the average excretion by adult men (*72*). Hormone and estrogen replacement therapies are other sources of estriol, estrone and estradiol in raw sewage. Thus, approx. 65 % and 15% of orally administered estradiol or estrone are excreted in urine and faeces, respectively.

Hormone contraceptives (birth control pills) contain ethinylestradiol, which is found mainly as sulphate conjungates (80 %) in plasma shortly after administration. A large part is excreted in un-metabolised but conjungated form. A total excretion of 26 % has been assumed (*72*). The total human

excretion of estrogens in Denmark (Table 1) was calculated using excretion patterns of estrogens and the demographic figures for Denmark 2001 (*75*).

Table 1 Estimated excretion of estrogens in Denmark 2001

G/24 h	Natural excretion	Excretion from hormone therapy	Excretion from hormone contraceptives	Total excretion
Estradiol	23.3	12.3		35.7
Estrone	53.1	15.5		68.6
Estriol	312	27.6		339.8
Ethinylestradiol			3.2	3.2

Potential sources of alkylphenols to sewage

Commercially available alkylphenols are generally mixtures of alkylphenols with different degrees of branching but with the same number of C-atoms in the alkyl chain (*76*). Alkylphenols are mainly used in the production of alkylphenolethoxylates (APnEO), tris(nonylphenyl)phosphite and alkylphenol-formaldehyde condensation resins (*77*). However, unreacted alkylphenols can be used as plasticisers in plastics. Alkylphenolethoxylates are relatively easily degraded to alkylphenols and, therefore, important sources of alkylphenols (*78*). Nonylphenol is the most commercially prevalent of the alkylphenol family, representing approx. 85 % of the alkylphenol market. The remaining 15 % are assumed to be octylphenol.

Nonylphenolethoxylates and nonylphenol may be released from formulation and from use in Denmark in e.g. cleaning processes. Estimates for the release to waste water in Denmark have been calculated on the basis of the EU risk assessment of nonylphenol (*76*) and figures for former Danish releases, where available. The Danish release is estimated to between 37 and 996 t/year[1].

Potential sources of bisphenol A to sewage

The major consumption of bisphenol A is as a chemical building block in the production of polycarbonate plastic and epoxy resins accounting for approx. 71 % and 25 %, respectively, of the total use in the EU (*79*). The Danish release is, with some uncertainty, estimated to 735 kg/year.

General considerations regarding establishment of the fate of estrogens and estrogenic compounds in sewage treatment plants

Evaluation of the fate of estrogens, alkylphenols, the parent compounds of alkylphenols and bisphenol A in municipal sewage treatment plants (STPs) show that a considerable amount of the studies presented in the literature only includes analyses of effluents. Thus, only few studies include simultaneous monitoring of influents, sewage samples and intermediate streams in STPs, which are needed for a proper assessment of the fate of a compound within a STP. The most extensive investigations are performed of nonylphenolethoxylates (NPnEO). Furthermore, there are only very few studies of Danish STPs. Another drawback is that variable procedures of sampling have been used from one study to another, and in some cases within the same study. Also the applied analytical methods including their limits of

[1] The Danish EPA made a voluntary agreement with 'The Association of Danish Cosmetics, Toiletries, Soap and Detergent industries (SPT)' in 1987 concerning a reduction of the use of alkylphenols. The present release depends on the success of this agreement.

detection and determination vary between different studies. All these things limit the possibilities of an exhaustive evaluation of the fate of the compounds in STPs and of an assessment of the impact of different treatment processes. Despite these facts it has been possible to extract some general aspects concerning the fate of the compounds of concern in STPs and the impact of the type of treatment plants on the removal efficiencies.

Fate of estrogens in sewage treatment plants

The estrogens are mainly excreted as conjugates. However, there are only very few studies which have included analysis of conjugated estrogens. Laboratory experiments have shown that the glucuronide conjugates of estradiol may be deconjugated relatively fast in suspensions of activated sludge (*80*) (*81*). It has been questioned whether these inactive conjugates are cleaved in the STP and perhaps already in the raw sewage and thereby released to the environment as active estrogens. An examination of both un-conjugated and conjugated estrogens (estradiol, estrone and ethinylestradiol) in raw sewage and sewage effluent from STPs in Germany showed that the conjugates contributed with up to approx. 50 % of the total steroid concentration in raw sewage containing 25.5 ng estrogen/l (*82*). The concentration of the total estrogens was reduced during the treatment process giving a total median estrogen concentration of 9.3 ng/l. However, the concentration of conjugates were still relatively high with a median value of 6.3 ng/l.

Investigations of the aerobic transformation of natural estrogens in activated sludge have shown that the possible initial degradation steps of a glucuronide conjugate of estradiol are: Estradiol-glucuronide → estradiol → estrone

The conjugates of estradiol seem to be de-conjugated relatively fast and the estradiol oxidised into estrone that is further eliminated. Biodegradation studies of estrogens with activated sludge form different sources showed a higher removal rate in sludge from a municipal STPs compared to the removal rate in sludge from an industrial STPs. This confirms the importance of an adapted microbial population in the biological removal of estrogens. Determination of mineralisation rates of (4-^{14}C)-estradiol in aerated activated sludge from STPs in Måløv, Denmark showed a first order rate constant of 0.031 ± 0.003 l/d/g suspended solid (SS) at 15 °C when the concentration was less than 2.5 µg/l. No significant degradation of (4-^{14}C)-estradiol was observed in an anoxic test system with the same type of sludge. Average sludge distribution coefficients K_d for ^{14}C labelled compounds in the aerobic and anoxic test system with Måløv sludge were estimated to 0.25 l/g SS and 0.96 l/g SS, respectively (*83*). The mineralisation rate of ethinylestradiol is low compared to estradiol. Degradation of both compounds has been seen at temperatures down to 5 °C but the rate was significantly reduced at low temperature (*84*).

Investigations of samples taken from either the influent or the effluent from primary sedimentation tanks and final effluent from STPs in Canada, Germany, Italy, the Netherlands, Brazil and Japan show that the concentrations of estrogens are within the same ranges (*85*) (*86*) (*87*) (*88*) (*89*). The removal of estradiol and estriol are generally more extensive than the removal of estrone and ethinylestradiol. The removal efficiency of the four estrogens in the study of the Italian STPs, i.e. the percentage removal from the water phase, showed following average removal efficiencies: 61 ± 38 % of estrone, 87 ± 9 % of estradiol, 96 ± 6 % of estriol and 85 ± 14 % of

ethinylestradiol (*87*). It is not possible to make any conclusion concerning the amount removed by biodegradation. It is obvious from the results obtained in batch tests that removal of the estrogens from the water phase in STPs may occur by degradations as well as sorption to sludge particles.

Fate of alkylphenols in sewage treatment plants

The alkylphenols (AP) in STPs are mainly a result of the biodegradation of alkylphenolethoxylates (APnEO). The degradation of APnEO is initiated by sequential cleaving of ethoxylated units. Under aerobic conditions the resulting products are alkylphenols, mono- and diethoxylates and the more hydrophilic carboxylates. Carboxylation may occur in both the alkyl and ethoxy side chains of the molecules. AP seems to be degradable under aerobic conditions. Thus, no detectable amounts of NP and OP were found after 35 days in a batch test inoculated with activated sludge from a STP in USA (*90*). The transformation of APnEO under anaerobic conditions (oxygen free conditions) as under anaerobic digestion of sludge results in production of mono- and diethoxylates and finally APs. APs are not further degraded under anaerobic conditions (*91*). This results, generally, in extremely high concentration of APs in anaerobically digested sludge, which may be reduced by introducing a post-aeration step (*92*). The mono- and diethoxylates as well as APs are hydrophobic compounds, which also are removed from the water streams within STPs by sorption to sludge particles.

Examinations of the fate of NPnEOs and their metabolites inclusive mono- and dicarcarboxylates have showed average elimination of nonylphenolic compounds (NP-c) from the water phase of 53 % and 59 % in traditional STPs mainly consisting of primary sedimentation, activated sludge processes, secondary sedimentation and anaerobic digestion (*93*) (*94*). The primary sedimentation did not significantly alter the distribution between the different metabolites (alkylphenol, alkylphenolethoxylates and carboxylated alkylphenols (APnEC)). However, APnEC was the most abundant group of the metabolites in the effluent after the activated sludge treatment. Investigations of eleven STPs in Switzerland showed that approx. 20 % of the amount of NP-c let to the STPs ended up in the digested sludge. Approx. 40 % was released with the effluent to the recipient (*93*).

There are no studies on the fate of APnEOs within STPs in the evaluated literature, which include all the known metabolites of APnEO and there might be even more not yet identified metabolites (*95*). Therefore, the potential pool of AP in the effluent from STPs may be considerably higher than known today.

Fate of bisphenol A in sewage treatment plants

Biodegradation studies of bisphenol A have shown that the compound should be easily degraded under aerobic condition in the activated sludge tank of STPs. However, degradation under anaerobic or anoxic conditions is much more unlikely to occur. It can also be expected that an amount of the bisphenol A received by STPs will be removed from the water phase by sorption. Examinations of samples from influent, effluents and sludge of Canadian STPs showed removal efficiencies from the water phase of 47-96 %. Concentration in samples of digested sludge were of 316-12,500 ng/g dry weight (*96*).

Influence of the type of sewage treatment plant on the removal efficiency of the estrogens/estrogenic compounds

The fate of the compounds in STPs is not only correlated with the intrinsic properties of the compounds, i.e. physico-chemical properties and degradability but also the type of sewage treatment process and the operation conditions. However, treatment conditions of the STPs studied are often not completely described. Hydraulic retention time (HRT), sludge retention time (SRT), temperature, denitrification, nitrification, and phosphate elimination will all have an important bearing on the plants efficiency (*95*). Comparing data within single studies, in which the sampling techniques and analytical methods are identical, was thought to give the best basis for evaluation of the importance of different treatment processes. The present assessment of the effects of the type of STPs was therefore mainly based on studies with investigations of STPs with different processes.

Generally, the effluent concentrations of estrogens and alkylphenol compounds (AP-c) seem to decrease by upgrading the STPs to nutrient removal and by using other tertiary treatment process as e.g. tertiary lagoons, micro-filtration, reverse osmosis and ultra violet light (UV-light). E.g. effluent concentrations of < 0.1-0.32 ng estradiol/l have been observed in Californian STPs after reverse osmosis (*97*). Danish investigations of both low and high technology STPs in Århus showed that the lowest effluent concentration of both estrogens and alkylphenolic compounds (AP, AP1EO, AP2EO) generally were found in the high technology plants (*98;99*). The highest effluent concentration was found in a STPs with only mechanical treatment, which also showed the lowest observed removal efficiency of bisphenol A (20 – 38 %). However, effluent concentrations of nonylphenolic compounds were seen at the same level in one of the high technology plants, Søholt STP. Analysis of influent samples from this STP showed correspondingly high concentrations. Identification of the sources of NPnEOs in the catchment area showed that 52 % of the NPnEOs
(n = 0-15) in the influent were discharged from a single industry. The concentrations of NPnEO (n = 0-2) in the effluent from the other high technology plants were in the range of approx. 0.2 to 1.2 µg/l. It can be concluded that the use of low technology plants, especially plants of very low technology in the so-called "open land" can be expected to result in relatively high concentrations of estrogens and xenoestrogens in the mixing zone of a recipient compared to plants of higher technology.

The highest reported effluent concentration of bisphenol A was found in the Danish Randers STP at 4,000 ng/l. The plant is upgraded to nutrient removal. It has not been possible to explain this high concentration on the basis of the available data (*98*).

Only few of the investigators have reported HRT and SRT of the studied STPs. However, long retention in the biological treatment step should increase the time of degradation. Furthermore, a long SRT of e.g. 25 days compared to a short retention time of e.g. 5 days may increase the possibility of establishing and maintaining a microbial flora capable of degrading the estrogens and xenoestrogens. The effect of HRT and SRT cannot be evaluated on the basis of the available data in the literature. However, an investigation of influent and effluent samples from three Dutch STPs based on the activated sludge system showed that the highest removal efficiencies of estrone and estradiol were obtained in the two plants with the highest HRTs (18 and 26 h) and SRTs (11 and 20 d) (*88*).

Apparently, there is a tendency to higher concentrations of especially estrogens in effluents from the U.K. compared to the other European countries. However, the data in the literature do not allow any final conclusions regarding potential differences between countries.

Influence of advanced sewage treatment processes on removal efficiencies

Conventional sewage treatment is not an effective barrier to trace contaminants like estrogens and xenoestrogens. There has, therefore, been an increasing focus on the use of more advanced treatment processes with the objective to remove the contaminants. Pilot scale studies have showed that both ozone/hydrogen peroxide and reverse osmosis effectively reduced estrogenicity of the secondary effluent from a full scale STP. No significant differences were observed using sand filtration or microfiltration on the same type of effluent (*100*). Laboratory experiments with estrone, estradiol, and ethinylestradiol standards showed removal in the range of 4-24 % after UV-treatment, and application of activated carbon (50 mg/l) has resulted in mean removal of estrogens from a pilot plant of 94.4 % (*101*). Two ongoing three years projects both initiated in 2000 are testing methods with the object of removing endocrine disrupters:

- An Australian project: "Optimised Use of Membrane Hybrid Processes for Water Recycling" (ARC SPIRT Project) (*102*)
- The EU-project POSEIDON working with the development of possible clarification techniques for increasing the removal of endocrine disrupters (*103*).

The key findings in the Australian project until now have been a negligible removal (< 10 %) of estrone with ferric chloride coagulation and very high removal (> 90 %) with powdered activated carbon. Magnetic ion exchange varied from 40 to 70 % removal depending on solution chemistry and dissociation of the hormone. Nanofiltration showed an initial retention of 70-95 % but, for most membranes, this retention dropped significantly after an initial filtration period. For some reverse osmosis membranes, retention was similar to nanofiltration, but others showed a very high and stable retention of the compounds. Microfiltration also showed initial almost complete retention followed by a drop as expected. The obtained results are currently being confirmed on larger scale systems (*102*).

The application of ozone and UV treatment of effluent from STPs is studied in the EU project POSEIDON. The use of an initial ozone treatment has been seen to improve the conditions for a following UV-treatment by decreasing absorption coefficients, which eventually improves the UV transmittance from 59 % to 84 %. The preliminary results of a pilot-scale process show a positive treatment effect regarding endocrine disrupters. The operating costs of the production of ozone were estimated to be between 0.90 and 1.60 EURO per kg ozone depending on the energy prices and the system capacity (*103*).

It can be concluded that more knowledge concerning the fate of both estrogens and xenoestrogens within STPs are needed. There should be high quality studies of influent, effluent, sludge and internal streams of STPs using standardized sampling procedures and validated analytical methods. The studies shall be linked to concurrent comprehensive monitoring of overall STP performance e.g. loading rate, removal efficiency of nutrient, HRT and SRT, as already suggested by Johnson et al. (2001). STPs with different treatment processes should be studied with the purpose of understanding the

influence of different process designs and operation criteria. The monitoring of full-scale plants should be accompanied by studies in pilot- and laboratory-scale.

Possible non-sewage effluent related sources of estrogens to the aquatic environment
Besides sewage effluent other sources of estrogens to the aquatic environment might have to be considered. Two potential sources are the use of manure from life stock and sludge from sewage treatment plants as fertilisers on fields. Insufficient knowledge exist, however, on the extent of runoff of estrogens with drain water and it is not possible yet to assess whether these are considerable sources of estrogenic activity to the surface waters.

2 Resumé (dansk)

Feminisering af hanfisk i ferskvandsmiljøet og det marine vandmiljø
Gennem de seneste ti år har man fundet feminisering af hanfisk i en række europæiske lande samt i USA og Japan (*1-12*). Disse tilfælde er eksempler på forstyrrelser af det hanlige forplantningssystem, som menes at være en konsekvens af hormonforstyrrelser forårsaget af hunlige hormoner, østrogener, eller østrogenlignende kemikalier, som er til stede i det akvatiske miljø. Størstedelen af østrogenerne formodes at nå det vandige miljø med spildevandsudledninger. I Europa er feminisering af hanfisk udsat for spildevand set i England (*1*), Sverige (*3*), Norge (*4*), Tyskland (*5*), Holland (*6*), Frankrig (*7;13*), Spanien (*8;14*) og Danmark (*2*). Desuden har man gjort observationerne af feminisering blandt vildtlevende fiskebestande hos flere arter incl. ferskvandsarterne skalle (*1*), grundling (*15*), karpe (*8;14*), brasen (*5;6*), døbel (*7;13*) og bækørred (*2*) samt saltvandsarterne skrubbe (*16-22*), ålekvabbe (*18*) og to arter af sandkutling (*23*). Disse typer af forstyrrelser i det hanlige forplantningssystem er således både blevet fundet i det ferske og marine vandmiljø, skønt de fleste tilfælde er blevet rapporteret fra ferskvandsmiljøet. Kontrollerede eksponeringer af fisk for spildevand i f.eks. burforsøg har yderligere givet evidens for den østrogene aktivitet i spildevand og østrogener og/eller østrogene stoffer som årsag til forstyrrelserne.

Tegnene på feminisering i hanfisk er dannelsen af et hunligt blommeprotein, som kun dannes som respons på en østrogen påvirkning, samt forekomsten af intersex – en unormal form for tvekønnethed. Hanner, der udviser intersex, har tidlige stadier af ægceller i testiklerne, og i nogle tilfælde har de også udviklet den hunlige struktur, som leder æggene til æggelederen. Feminisering er blevet fundet i forskellige udviklingsgrader blandt fisk fra milde til alvorlige forstyrrelser af det hanlige forplantningssystem.

England skiller sig ud som det land, hvor feminisering af fisk er mest udbredt, og hvor de mest alvorlige grader af feminiseringer er observeret – både sammenlignet med lande i og udenfor Europa. Den feminisering af fisk, som er blevet fundet i Danmark afviger dog ikke markant fra det omfang, der er set i de øvrige lande uden for England. I alle lande, hvor undersøgelser er foretaget, er der fundet lokaliteter, hvor fiskepopulationer er upåvirkede.

Det skal pointeres, at korttids-eksponeringsforsøg, hvor fisk kun udsættes for spildevand for en kort periode, ikke altid giver et korrekt estimat af risikoen for vilde populationer af fisk, som lever hele deres liv i det pågældende vandløb. Det er påvist, at en øget eksponeringstid for spildevand nedsætter den koncentration af spildevand, som kræves for at forårsage feminisering af fisk (*24*).

Østrogenicitet af spildevand
Teorien om, at østrogener/østrogene stoffer i spildevand er årsag til feminiseringen af hanfisk, støttes også af en række forsøg, hvor der er anvendt cellekultur-testsystemer udviklet til at detektere østrogen aktivitet. Disse har demonstreret østrogen aktivitet i spildevand fra en række lande (England, Tyskland, Holland, Belgien, Kina, Korea og USA) og har kvantificeret spildevandets østrogene potentiale i forhold naturligt østrogen, 17ß-østradiol

(*6;25-33*). Kemiske analyser af spildevandets sammensætning og fastsættelse af koncentrationer af østrogener og østrogene stoffer i spildevandet har yderligere demonstreret, at de naturlige østrogener, 17ß-østradiol, østron og østriol samt det syntetiske østrogen, ethinyløstradiol – anvendt i p-piller - i flere tilfælde var de sandsynlige kandidater, når årsagen til de observerede forstyrrelser blandt fisk fra de spildevandsbelastede floder skulle fastsættes. I enkelte tilfælde er de østrogene kemikalier, alkylphenoler, også blevet udnævnt som mulige ansvarlige stoffer for feminiseringen af hanfiskene (*34*).
I de tilfælde, hvor man har kombineret kemisk analyse af spildevand med vurdering af den samlede østrogenicitet (bestemt i cellekultur-testsystemer) i spildevandet, har man været i stand til at redegøre for den samlede østrogenicitet ved hjælp af de fundne stoffer (naturlige østrogener, ethinyløstradiol, alkylphenoler, bisphenol A). Det er således vigtigt at slå fast, at der ikke i de nævnte undersøgelser er er behov for at inkludere ukendte kemikalier med østrogen virkning for at forklare spildevandets samlede østrogenicitet.

Østrogenkoncentrationer i spildevand og overfladevand
Internationalt er de tre naturlige østrogener, 17ß-østradiol, østron og østriol fundet i spildevand i koncentrationerne < 0,1 – 88 ng/l, < 0,1 – 220 ng/l and < 0,1 – 42 ng/l. Det endnu mere potente syntetiske østrogen, ethinyløstradiol, er fundet i koncentrationerne < 0,053 – 62 ng/l. De typiske koncentrationer ligger dog indenfor intervallerne 1 – 10 ng/l og 5 – 20 ng/l for hhv. 17ß-østradiol og østron, mens ethinylestadiol ofte er under detektionsgrænsen for analysemetoderne (0,1-1 ng/l). Når ethinyløstradiol detekteres, er det oftest under 10 ng/l. De koncentrationer, der er målt i dansk spildevand ligger indenfor de østrogenkoncentrationer, som er rapporteret fra andre europæiske lande.
I overfladevand er østrogenkoncentrationerne lavere end i spildevand og er detekteret i intervallerne 0,05 – 15,5 ng østradiol/l, < 0,1 – 17 ng østron/l, < 0,1 – 3,4 ng østriol/l and < 0,053 – 30,8 ng ethinyløstradiol/l med typiske koncentrationer under 5 ng/l for østradiol og østron og mindre end 1 ng/l for ethinyløstradiol. I både spildevand og overfladevand er østron det hyppigst forekommende og ethinyløstradiol det sjældnest forekommende af østrogenerne.

En vigtig ting, man skal være opmærksom på, når der nævnes østrogenkoncentrationer i nærværende rapport, er, at det sjældent er specificeret, hvorvidt de kemiske analyser kun måler frie eller både frie og konjugerede østrogener. Man må også formode, at der er større usikkerheder forbundet med østrogenmålinger i indløbs- end udløbsspildevand primært pga. interferens fra store mængder organisk stof (matrix effekt). Desuden er detektionsgrænsen for analyserne – især for ethinylestradiol – tæt på og nogle gange endda over de koncentrationer, man har vist kan forårsage kønsforstyrrelser hos fisk.

Østrogeners skæbne i det vandige miljø
Vor viden om østrogeners skæbne, adfærd og persistens i miljøet er stadig relativt begrænset. Det syntetiske ethinyløstradiol er dog tilsyneladende mere persistent end de naturlige østrogener både i vandfasen og i sediment. Under tilstedeværelse af ilt er den gennemsnitlige halveringstid i vand for 17ß-østradiol og østron beregnet til hhv. 2,8 og 3,0 dage (*35*). Under ens inkuberingsforhold er det påvist, at halveringstiden for ethinyløstradiol er ti gange så lang som for 17ß-østradiol (1,2 versus 17 dage for hhv. østradiol og ethinyløstradiol) (*35;36*). Østrogener har generelt en medium sorptionsevne

til sediment (*37*). I iltfrie sedimenter omsættes 17ß-østradiol forholdsvis hurtigt til østron, som i lighed med ethinyløstradiol under disse betingelser nedbrydes langsomt eller slet ikke. Den ringe nedbrydning af østron og især ethinyløstradiol i sediment med lav iltningsgrad indikerer evt. en risiko for, at sedimentet akkumulerer disse østrogener (*35*) .

Forkomst og skæbne af udvalgte østrogene stoffer i spildevand og overfladevand
Alkylphenoler og bisphenol A hører til de mere potente af de østrogene kemikalier, som kan frigives med spildevand, og som derfor er omtalt i denne rapport. De vigtigste alkylphenoler mht. østrogenicitet er nonylphenol og oktylphenol. Disse er generelt detekteret i koncentrationer under 10 µg/l i spildevand, skønt der er eksempler på koncentrationer over 300 µg/l i spildevand og over 600 µg/l i flodvand fra nogle lande. Bisphenol A er mindre hyppigt forekommende og er sjældent målt i koncentrationer over 1 µg/l i spildevand og overfladevand. Som for østrogener fordeler alkylphenoler og bisphenol A sig mellem vandfasen og sedimentet, og der er indikationer for, at begge har et stort potentiale for at akkumulere i sediment med ringe iltforhold (*38;39*).

Østrogeners, alkylphenolers og bisphenol A's feminiseringspotentiale
I lyset af de hormonforstyrrelser, som er observeret blandt fiskepopulationer, som har været udsat for spildevandspåvirkning, er der blevet udført et antal kontrollerede eksponeringsforsøg for at fastsætte de laveste koncentrationer af østrogener og østrogene kemikalier, som kræves for at fremkalde disse forstyrrelser. Dette er en hjælp til at fastslå hvilke stoffer, der kan være ansvarlige for de observerede effekter som dannelse af blommeprotein hos hanner, udvikling af intersex og andre forstyrrelser af hanners testikeludvikling.
17ß-østradiol har forårsaget dannelse af blommeprotein ved en koncentration på 5 ng/l (*40;41*) og har forårsaget udvikling af intersex ved 10 ng/l (*42*), og en række andre testikeleffekter er set ved koncentrationer mellem 10 – 50 ng/l (*43-45*) og også ved koncentrationer over 100 ng/l (*44*). Eksempler på andre testikeleffekter er hæmning af den normale udvikling af hanlige kønsceller, som kan ses som en lavere testikelvægt i forhold til kropsvægten og/eller en tilstedeværelse af en større andel af tidlige, mindre modne stadier af kønsceller (*44*). Forekomst af nedbrudte kønsceller er også set (*44*).

En lignende men lidt lavere østrogen aktivitet er set for østron. Dannelse af blommeprotein og fremkaldelse af intersex i hanfisk er set ved koncentrationer på hhv. 30 (*46;47*) og 10 ng/l (*42*). Østriol er det mindst østrogene af de tre naturlige østrogener. *In vitro* har det en 30 gange lavere potens end østradiol, men mht. udvikling af intersex *in vivo* er det tilsyneladende 100 gange mindre potent (*42*). Generelt er der begrænset viden om den østrogene kapacitet af dette østrogen.

Ethinyløstradiol er mere potent end de naturlige østrogener mht. at forårsage forstyrrelser i det hanlige forplantningssystem. Dannelse af blommeprotein og udvikling af intersex er set ved 0,1 ng/l (*42*) og ændret kønsratio ved 0,6 ng/l (*48*). En række andre effekter på forplantningssystemet som hæmning af den normale sædcelleudvikling er set ved koncentrationer under 10 ng/l.

Både alkylphenolerne, nonylphenol samt oktylphenol og bisphenol A har svagere østrogen aktivitet end både naturlige og syntetiske østrogener. Effekter er set ved koncentrationer i µg/l-området. Dannelse af blommeprotein i hanfisk er set ved en koncentration på 5 µg/l nonylphenol eller oktylphenol

(*49;50*), skønt langtidseksponering for nonylphenol sænkede den laveste effekt koncentration til 1 µg/l (*51;52*) . Intersex, ændret kønsratio, nedbrudt testikelvæv og hæmmet vækst af testikler er set ved nonylphenol koncentrationer mellem 30 og 100 µg/l (*50;53;54*). Koncentrationer ned til 2 µg/l oktylphenol har forårsaget forstyrrelser hos hanfisk (*49*). Bisphenol A har udvist effekter ved koncentrationer mellem 10 og 40 µg/l (*42;55;56*).

Sammenhæng mellem effektkoncentrationerne af østrogener/østrogene stoffer og deres tilstedeværelse i miljøet

Når man sammenligner de aktuelle koncentrationer af østrogener, alkylphenoler og bisphenol A med de laveste koncentrationer, som i kontrollerede laboratorieforsøg har kunnet forårsage hormonforstyrrelser i hanfisk, fremgår det, at koncentrationerne af 17ß-østradiol, østron og ethinyløstradiol i nogle tilfælde har været tilstrækkelig høje til at forklare feminiseringen af fisk i vandløb. Det samme gælder i nogle tilfælde for nonylphenol og oktylphenol. Der er for få data på miljøkoncentrationer af østriol til at kunne give et troværdigt estimat for dette østrogens mulige bidrag til feminiseringseffekter, mens bisphenol A generelt er blevet fundet i koncentrationer under den laveste effektkoncentration for at fremkalde forplantningsforstyrrelser hos hanfisk.

I Danmark er der kun foretaget relativt få målinger af østrogenkoncentrationer i vand, og det er endnu ikke muligt entydigt at udpege de/det ansvarlige stof(fer) for de observerede feminiseringer af hanfisk i danske vandløb (*2*).

Når man skal vurdere de mulige betydninger af østrogene stoffer i det vandige miljø for forplantningssystemet hos hanfisk, er det i øvrigt vigtigt at huske, at østrogenerne i vandet vil virke additivt (*40;57*). Derfor vil koncentrationen af et enkelt stof, som kan udøve en effekt på hanner, være lavere, når stoffet er til stede i en blanding af østrogener og østrogene stoffer. Desuden udviser forskellige arter af fisk forskellig følsomhed overfor hormonforstyrrelser skabt af østrogener, og timingen af eksponeringen er også vigtig for de resulterende effekter. De tidlige livsstadier betragtes generelt som de mest sensitive stadier, da udviklingen af kønnet foregår i denne periode (*58*). Der kan dog også forekomme perioder i forplantningscyklen hos kønsmodne hanfisk, i hvilke disse er mere sårbare overfor en hormonforstyrrelse. Påvirkning med pulser af høje koncentrationer af østrogener har yderligere vist sig at resultere i større effekter end de, som opnås ved en kontinuert påvirkning med lavere koncentrationer (*59*). Frigivelse af korte pulser med meget høje koncentrationer af østrogener og/eller østrogene stoffer kan derfor være af stor betydning.

Effekten af feminisering eller østrogen eksponering for fertiliteten hos han- og hunfisk

Observationerne af feminiserede fisk i mange dele af verden har rejst spørgsmålet, hvorvidt frugtbarheden eller forplantningsevnen hos fisk er nedsat, og om der er risiko for fald i bestandenes størrelse. Baseret på den nuværende viden er det ikke muligt at svare. Nye resultater har dog indikeret nedsat befrugtningsevne blandt intersex-skallehanner i England (*57;60*). En asynkron udvikling af kønsceller mellem hanner og hunner er blevet set pga. en forsinkelse i udviklingen af hanlige kønsceller. Kun 50 % af hannerne var i stand til at gyde. Yderligere er der set et nedsat volumen af sæd og en nedsat sædcelletæthed og –bevægelighed blandt de øvrige hanner. Dette indikerer nedsat befrugtningsevne blandt hanner med udpræget udvikling af intersex. Betydningen af en mildere grad af feminisering er sværere at estimere.

Kontrollerede forsøg med østrogener og østrogene stoffer har også demonstreret en nedsat befrugtningssucces samt en ændret sexual adfærd hos eksponerede hanner (*64-67*).

De fleste undersøgelser både i naturen og i laboratoriet har fokuseret på effekter af østrogener og østrogene stoffer på den forplantningsmæssige sundhedstilstand hos hanfisk, da disse betragtes for at være det mest følsomme køn pga. deres lave kropskoncentrationer af østrogen. Der er imidlertid undersøgelser, som også har vist, at forplantningssuccessen hos hunner kan være reduceret ved en østrogeneksponering f.eks. ved nedsat gydning af æg. Dette foregår tilsyneladende via en forstyrrelse af den normale ægmodning (*61;68;69*).

Potentielle kilder til østrogener i spildevand

De tre østrogener østradiol, østron og østriol er hunlige kønshormoner, som produceres naturligt i mennesker og andre vertebrater. Østrogenerne produceres både af hun- og handyr. Produktionen og udskillelsen varierer igennem livet og er endvidere forskellig hos de to køn. Østradiol metaboliseres både reversibelt og irreversibelt. I den reversible proces omdannes østradiol til østron, mens østradiol omdannes til catecholøstrogener eller østriol i den irreversible metabolisme. Hovedparten af de producerede metabolitter konjugeres[2] efterfølgende med sulfat eller glukuronider, hvorefter de udskilles i urinen. De udgør dermed en vigtig kilde til forekomsten af naturlige østrogener i det kommunale kloaksystem. En mindre del af kønshormonerne udskilles via fæces i form af ukonjugerede metabolitter (*70;71*).

Østrogenproduktion hos den voksne mand varierer med alderen, men variationerne er små i sammenligning med variationen hos kvinder. Kvinders østrogenproduktion og –udskillelse varierer gennem menstruationscyklus og eventuel graviditet indtil menopausen. Menstruerende kvinder udskiller dagligt i gennemsnit: 4,8 µg østriol, 8,0 µg østron og 3,5 µg østradiol via urinen. Den daglige gennemsnitlige udskillelse for gravide kvinder via urinen er: 6,000 µg østriol, 600 µg østron og 259 µg østradiol (*72*) (*73*). Østrogenproduktionen aftager ved menopausen, hvorefter østrogenproduktion hos kvinder er meget lav (*74*). Efter denne periode udskiller kvinder i gennemsnit ca. 7 µg af de tre østrogener pr. dag. Dette svarer til den gennemsnitlige daglige udskillelse fra voksne mænd (*72*).

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[2] Kemikalier, der har en høj fedtopløselighed, er generelt svære for organismer at udskille fra kroppen i deres oprindelige form, mens vandopløselige stoffer lettere udskilles. Organismer har imidlertid udviklet enzymsystemer, der er i stand til at øge vandopløseligheden af ellers fedtopløselige kemikalier. Den ene mulighed er, at organismen omsætter og ændrer det oprindelige kemikalie. Den anden mulighed er, at organismen kobler et vandopløseligt stof på det fedtopløselige kemikalie (en såkaldt konjugering), sådan at vandopløseligheden af det dannede konjugat bliver større end vandopløseligheden for det oprindelige kemikalie. Både naturligt dannede stoffer som fedtopløselige hormoner (f.eks. østrogener) og fedtopløselige industrikemikalier kan blive gjort mere vandopløselige ved konjugationsreaktioner.
Blandt den gruppe af vandopløselige stoffer, som organismen kan lade indgå i konjugationsreaktioner, er sulfat og glucoronsyre de mest betydende med hensyn til udskillelse af østrogener. Det er vigtigt at bemærke, at konjugatet af østrogen + sulfat eller glukoronsyre ikke har hormonaktivitet. De østrogenkonjugater, der udskilles fra mennesker, kan imidlertid igen spaltes af bakterier i spildevand og i rensningsanlæg, således at det fri, aktive østrogen gendannes.

Hormon- og østrogenterapeutiske præparater er andre kilder til østriol, østron og østradiol i spildevand. Af de mængder østradiol eller østron, som indtages oralt, udskilles således ca. 65 % og ca. 15 % via urin og fæces.

Antikonceptionelle hormonmidler (svangerskabsforebyggende piller) indeholder ethinyløstradiol, som optræder i plasmaet kort efter indtagelse i form af sulfat-konjugater (80 %). En væsentlig del (ca. 26 %) af den indtagne mængde ethinylestradiol udskilles på konjugeret form (*72*).

Den totale humane østrogenudskillelse i Danmark blev estimeret ved anvendelse af udskillelsesmønsteret for østrogener samt demografiske data for Danmark fra 2001 (*75*). De estimerede mængder er vist i tabel 1.

Tabel 1 Estimeret udskillelse af østrogener i Danmark 2001

g/døgn	Naturlig udskillelse	Udskillelse fra hormonterapi	Udskillelse fra antikonceptionelle hormonmidler	Total udskillelse
Østradiol	23,3	12,3		35,7
Østron	53,1	15,5		68,6
Østriol	312	27,6		339,8
Ethinyløstradiol			3,2	3,2

Potentielle kilder til alkylphenoler i spildevand

Kommercielt tilgængelige alkylphenoler er generelt blandinger af alkylphenoler med forskellig grad af forgrening, men med samme antal C-atomer i alkylkæden (*76*). Alkylphenoler anvendes hovedsageligt i produktionen af alkylphenolethoxylater (APnEO), tris(nonylphenyl)phosphite og alkylphenol-formaldehydkondensationsresiner (*77*). Alkylphenoler (AP) kan imidlertid også anvendes som blødgøringsmidler i plastik. Alkylphenolethoxylater nedbrydes relativt let til alkylphenoler og er derfor vigtige kilder til alkylphenoler (*78*). Nonylphenol er den mest udbredte af de kommercielle alkylphenoler og udgør ca. 85 % af alkylphenolmarkedet. De resterende 15 % er antageligt octylphenol.

I Danmark vil nonylphenolethoxylater og nonylphenol kunne udledes fra formulering og anvendelse i bl.a. regøringsprocesser. Udledningen til spildevand i Danmark er blevet estimeret på grundlag af EU's risikovurdering for nonylphenol (*76*) samt lidt ældre danske data for udledning, hvor det har været muligt. Den samlede udledning er estimeret til at være mellem 37 og 996 t/år[3].

[3] Miljøstyrelsen indgik i 1987 en frivillig aftale med Brancheforeningen SPT (Sæbe, Parfume og Teknisk kemiske produkter), om at reducere brugen af nonylphenolethoxylater i rengøringsmidler. Aftalen har medført at stofferne ikke længere anvendes af brancheforeningens medlemmer, hvilket dækker mellem 80 og 90% af markedet i Danmark. For at kontrollere om aftalen blev overholdt foretog Miljøstyrelsen i 1999 en analyse af indholdet af nonylphenolethoxylat og octylphenolethoxylat i 34 rengøringsmidler. Analysen viste, at den frivillige aftale, med en enkelt undtagelse, overholdes af såvel medlemmer af Brancheforeningen SPT som producenter udenfor brancheforeningen. Herudover blev det i 1995 aftalt mellem Miljøstyrelsen og Dansk Planteværn at udfase alkylphenolethoxylater i bekæmpelsesmidler, og der findes i dag ikke midler på markedet, der indeholder disse stoffer.
Vaske- og rengøringsmidler samt bekæmpelsesmidler udgjorde tidligere langt det største forbrug af alkylphenolethoxylat i Danmark. På dette grundlag antages at

Potentielle kilder til bisphenol A i spildevand
Hovedparten af bisphenol A anvendes som kemisk byggeblok i produktionen af polycarbonat og epoxyresiner. Forbruget hertil udgør henholdsvis 72 % og 25 % af det totale forbrug i EU (*79*). Udledningen af bisphenol A til spildevand i Danmark estimeres – med nogen usikkerhed – til 735 kg/år.

Generelle betragtninger vedrørende vurderinger af østrogeners og østrogene stoffers skæbne i renseanlæg
En betragtelig del af undersøgelserne af skæbnen for østrogener, alkylphenoler, moderstofferne af alkylphenoler samt af bisphenol A i kommunale renseanlæg, beskrevet i litteraturen, omfatter kun analyser af udløbsvand. Der er således kun få studier, som omfatter sammenhørende undersøgelse af indløb, udløb, slam og interne strømme i renseanlæg, hvilket er nødvendigt for en dybdegående vurdering af stoffernes skæbne i et renseanlæg. De mest omfattende undersøgelser er foretaget af nonylphenolethoxylater (NPnEO). Der er endvidere meget få undersøgelser af danske renseanlæg. En anden ulempe er, at de procedurer, der er anvendt til prøvetagning, varierer fra en undersøgelse til en anden og nogle gange endda inden for samme undersøgelse. Også de anvendte analysemetoder samt deres detektionsgrænser og grænser for kvantificering varierer. Alle disse forhold begrænser muligheden for en udtømmende evaluering af stoffernes skæbne i renseanlæg samt for at vurdere betydningen af forskellige renseanlægstyper.

Østrogeners skæbne i renseanlæg
Østrogenerne udskilles hovedsageligt i form af konjugater. Der er imidlertid kun meget få undersøgelser, som har inkluderet analyser af konjugerede østrogener. Laboratorieundersøgelser har vist, at glucuronerede konjugater af østradiol kan dekonjugeres relativt hurtigt i en suspension af aktivt slam (*80*) (*81*). Det har været diskuteret, om disse inaktive konjugater dekonjugeres i renseanlægget og måske allerede i kloaksystemet med det resultat, at der frigives aktive østrogener til miljøet. En undersøgelse af ubehandlet spildevand samt udløbsvand fra renseanlæg i Tyskland, der omfattede analyse af både ukonjugerede og konjugerede østrogener (østradiol, østron og ethinyløstradiol) viste, at de konjugerede østrogener udgjorde op til 50 % af den total østrogenkoncentration på 25,5 ng østrogen/l (*82*). Den totale østrogenkoncentration i vandfasen blev reduceret gennem renseprocessen til en koncentration på ca. 9,3 ng/l. Andelen af konjugater var imidlertid stadig høj og havde en medianværdi på 6,3 ng/l.

Den initiale nedbrydning af et glucuronid-konjugat i aktivt slam forløber sandsynligvis således, når der er ilt til stede: østradiol-glucuronid → østradiol → østron.

Omsætningshastigheden for estradiol var hurtigere i slam fra et kommunalt renseanlæg end slam fra et industrielt renseanlæg. Dette bekræfter betydningen af at anvende en adapteret mikrobiel population til biologisk fjernelse af østrogener. Bestemmelse af mineraliseringshastigheder for (4-^{14}C)-østradiol i beluftet aktivt slam fra Måløv renseanlæg i Danmark viste en førsteordenshastighedskonstant på 0,031 ± 0,003 l/d/g suspenderet stof (SS)

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forbruget af alkylphenolethoxylat er meget lille i Danmark. Dette bekræftes endvidere af målinger af nonylphenol i slam fra 2000.

ved 15°C for koncentrationer mindre end 2,5 µg/l. Der blev ikke observeret signifikant nedbrydning af (4-^{14}C)-østradiol i et anoxisk testsystem med samme slamtype. Gennemsnitlige slamfordelingskoefficienter K_d for det ^{14}C-mærkede stof blev estimeret til 0,25 l/g SS og 0,96 l/g SS for henholdsvis det aerobe og det anoxiske testsystem (*83*). Ethinyløstradiols mineraliseringshastighed er lav i sammenligning med hastigheden for østradiol. Nedbrydning af begge stoffer er registreret helt ned til en temperatur på 5°C. Nedbrydningshastigheden var dog reduceret væsentligt ved de lave temperaturer (*84*).

Undersøgelse af prøver udtaget af enten indløbet eller udløbet fra den primære sedimentation samt af udløbet fra renseanlæg i Canada, Tyskland, Italien, Holland, Brasilien og Japan viser, at koncentrationerne af de respektive østrogener ligger inden for de samme intervaller (*85*) (*86*) (*87*) (*88*) (*89*). Fjernelsen af østradiol og østriol er generelt større end fjernelsen af østron og ethinyløstradiol. I de italienske renseanlæg blev fjernelsesgraden for de fire østrogener, dvs. den procentiske reduktion af stofkoncentrationen i renseanlæggets udløb i forhold til koncentrationen i indløbet, fundet at være: 61 ± 38 % for østron, 87 ± 9 % for østradiol, 96 ± 6 % for østriol og 85 ± 14 % for ethinyløstradiol (*87*). Det er ikke muligt at vurdere, hvor stor en andel af denne fjernelse, der skyldes biologisk nedbrydning. Resultater, der er opnået i laboratorietest, viser, at fjernelse af østrogener fra vandfasen i et renseanlæg kan ske dels ved bionedbrydning og dels via sorption til slampartikler.

Alkylphenolers skæbne i renseanlæg

De alkylphenoler (AP), som forekommer i renseanlæg stammer hovedsageligt fra bionedbrydning af alkylphenolethoxylater (APnEO). Nedbrydningen af APnEO initieres ved sekventiel fraspaltning af ethoxylatenhederne. Under aerobe forhold er de resulterende produkter alkylphenol, mono- og diethoxylater samt de mere hydrofile carboxylater. Carboxyleringen kan ske i enderne af både alkyl- og ethoxylatkæden. Laboratorietests tyder på, at AP er nedbrydeligt under aerobe forhold. Der blev således ikke fundet detekterbare koncentrationer af NP og OP efter 35 dage i en batchtest, som var inokuleret med aktivt slam fra et amerikansk renseanlæg (*90*). Under anaerobe forhold ligesom i den anaerobe rådnetank på et renseanlæg nedbrydes APnEO til mono-og diethoxylater samt slutproduktet AP (*91*). AP nedbrydes ikke yderlige under anaerobe forhold, hvilket ofte resulterer i meget høje AP-koncentrationer i slam fra rådnetårne. Den høje koncentration af AP i denne type slam kan dog reduceres ved at introducere et efterbeluftningstrin (*92*). Mono- og diethoxylater samt AP er hydrofobe stoffer, som også fjernes fra renseanlæggets vandfase via sorption til slampartikler.

Undersøgelser af skæbnen af NPnEO og metabolitter inklusive mono- og dicarboxylater har vist procentiske fjernelser af den samlede mængde af nonylphenoliske stoffer (NP-c) på 53 % og 59 % i traditionelle renseanlæg. Der vil sige anlæg bestående af primær sedimentation og en aktiv-slamproces efterfulgt af en sekundær sedimentation og anaerob udrådning af slammet (*93*) (*94*). Den primære sedimentation ændrede ikke fordelingen mellem de forskellige metabolitter (alkylphenol, alkylphenolethoxylater og carboxylerede alkylphenoler (APnEC)). Efter behandlingen i aktiv-slamprocessen udgjorde APnEC imidlertid den største gruppe af metabolitterne i udløbsvandet. Undersøgelser af elleve renseanlæg i Schweiz viste, at ca. 20% af den tilledte mængde nonylphenoliske stoffer endte i det udrådnede slam. Ca. 40% blev udledt til den akvatiske recipient via renseanlæggets udløbsvand (*93*).

Der er ingen af de gennemgåede undersøgelser af APnEO's skæbne i renseanlæg, som har omfattet samtlige kendte metabolitter af ApnEO, og det kan ikke udelukkes, at der findes flere endnu uidentificerede metabolitter (*95*). Den potentielle 'pool' af AP i udløbet fra renseanlæg kan derfor være betragteligt større end hidtil antaget.

Bisphenol A's skæbne i renseanlæg
Undersøgelser af bisphenol A's bionedbrydelighed tyder på, at stoffet nedbrydes let under aerobe forhold i et renseanlægs aktiv-slamproces. Det er derimod mere usandsynligt, at der sker en nedbrydning af bisphenol A under anaerobe og anoxiske forhold. Det kan endvidere forventes, at en del af den stofmængde, som ledes til renseanlægget, vil blive fjernet fra vandfasen via sorption til slampartikler. Undersøgelser af prøver udtaget fra indløb, udløb og slam på canadiske renseanlæg har vist fjernelsesgrader på 47-96 % fra vandfasen. Koncentrationen i prøver af udrådnet slam blev målt til 316-12.500 ng/g tørstof (TS) (*96*).

Betydningen af renseanlægstypen på fjernelsen af østrogener og østrogene stoffer
Skæbnen af stofferne i renseanlæg er ikke kun afhængig af stoffernes iboende egenskaber, dvs. fysisk-kemiske parametre og bionedbrydelighed. Stoffernes skæbne afhænger også af renseanlægstypen og driftsforholdene. Desværre er procesforholdene for de renseanlæg, der er undersøgt ofte mangelfuld beskrevet. For eksempel kan hydrauliske retentionstid (HRT), slamalder, temperatur, denitrifikation, nitrifikation, og fosfatfjernelse alle have en vigtig betydning for anlæggets effektivitet vedrørende fjernelse af miljøfarlige stoffer (*95*). Sammenligning af data fra flere renseanlæg som er indsamlet indenfor en enkelt undersøgelse, hvor der har været anvendt samme prøveteknik, analysemetode etc., burde give det bedste grundlag for en vurdering af renseanlægstypens betydning. Den foreliggende vurdering af renseanlægstypens betydning er derfor hovedsageligt baseret på enkeltstudier, som har omfattet undersøgelse af renseanlæg med forskellige behandlingsprocesser.

Generelt synes koncentrationerne af østrogener og alkylphenoliske stoffer (AP-c) at aftage med stigende opgradering af renseanlæggene til fjernelse af næringssalte og ved introduktion af andre tertiære processer som f.eks. tertiære laguner, mikrofiltrering, omvendt osmose og ultraviolet lys (UV-lys). Udløbskoncentrationer for østradiol på <0,1-0,32 ng/l blev f.eks. observeret i et canadisk renseanlæg efter behandling med omvendt osmose (*97*). Danske undersøgelser af lav- og højteknologiske renseanlæg i Århus viste, at de laveste koncentrationer af både østrogener og AP-c (AP, AP1EO, AP2EO) generelt blev fundet i udløb fra højteknologiske anlæg (*98;99*). De højeste koncentrationer samt den laveste fjernelsesgrad for bisphenol A (-20 - 38%) blev fundet i udløbet fra et renseanlæg, hvor renseprocessen alene bestod af mekanisk behandling. På et af de højteknologiske renseanlæg (Søholt) blev der fundet en lige så høj koncentration af nonylphenoliske stoffer i udløbsvandet som i lavteknologiske anlæg. Analyse af en prøve udtaget fra indløbsvandet viste imidlertid en forholdsmæssig høj indløbskoncentration. En efterfølgende identifikation af kilderne til NPnEO i renseanlæggets opland viste, at 52 % af den tilledte mængde NPnEO (n=0-15) kom fra en enkelt virksomhed. NPnEO (n = 0-2) koncentrationen i udløbet fra de øvrige højteknologiske renseanlæg lå i intervallet ca. 0,2 til 1,2 µg/l. Det kan konkluderes, at lavteknologiske renseanlæg må forventes at give anledning til højere koncentrationer af østrogener og xenoøstrogener i blandingzonen i en

recipient end anlæg med høj teknologi. Dette vil ikke mindst være gældende for de anlæg med meget lav teknologi, der er placeret i det åbne land.

Den højeste af de rapporterede udløbskoncentrationer for bisphenol A på 4.000 ng/l er målt i det danske renseanlæg i Randers. Renseanlægget er opgraderet til næringssaltsfjernelse. Det har ikke været muligt at forklare denne høje koncentration ud fra de foreliggende data om anlægget (*98*).

Der er kun meget få angivelser af den hydrauliske opholdstid og slamalderen i de foreliggende undersøgelser af stoffernes skæbne i renseanlæg. Det kan imidlertid forventes, at en relativt lang opholdstid i det biologiske behandlingstrin vil medføre en bedre stoffjernelse, idet tiden til nedbrydningsprocessen er længere. Det kan endvidere forventes, at en høj slamalder på f.eks. 25 dage vil øge mulighederne for etablering og opretholdelse af en mikrobiel flora, som er i stand til at nedbryde østrogener og xenoøstrogener, i forhold til en kort slamalder på f.eks. 5 dage. Betydningen af den hydrauliske opholdstid og slamalderen kan ikke vurderes på grundlag af de tilgængelige data i litteraturen. En undersøgelse af indløbs- og udløbsprøver fra tre hollandske renseanlæg viser imidlertid, at de højeste fjernelsesgrader blev opnået i de to af renseanlæggene med de højeste hydrauliske opholdstider (18 og 26 timer) og slamaldre (11 og 20 dage) (*88*).

Der er tilsyneladende en tendens til optræden af højere koncentrationer af især østrogenerne i udløbene fra engelske renseanlæg i sammenligning med anlæggene i de øvrige europæiske lande. Der er imidlertid ikke tilstrækkeligt datagrundlag i litteraturen til at drage endelige konklusioner vedrørende forskelle i stofudledningen mellem de enkelte lande.

Betydningen af avancerede former for spildevandsbehandling for fjernelsen af østrogener

Konventionel spildevandsbehandling er ikke nødvendigvis en effektiv barriere mod stoffer som østrogener og østrogenlignende stoffer. Der er derfor en stigende fokusering på anvendelse af mere avancerede renseteknikker til fjernelse af denne type stoffer. Forsøg i pilotskala har vist, at både ozon/hydrogenperoxidbehandling og omvendt osmose kan reducere østrogeniciteten effektivt i sekundært udløbsvand fra et fuldskala renseanlæg. Anvendelse af sandfiltrering eller mikrofiltrering på den samme type udløbsvand gav derimod ingen signifikant fjernelse af østrogeniciteten (*100*). Laboratorieundersøgelser af UV-lys' indvirkning på fjernelsen af østron, østradiol og ethinyløstradiol viste fjernelsesgrader i området 4-24 %. Anvendelse af aktivt kul (50 mg/l) har resulteret i gennemsnitlige fjernelser af østrogener på 94,4 % i et pilotforsøg (*101*). To igangværende treårige projekter, som begge startede i 2000, tester forskellige metoder til fjernelse af hormonforstyrrende stoffer fra spildevand:

- Et australsk projekt: "Optimised Use of Membrane Hybrid Processes for Water Recycling" (ARC SPIRT Project) (*102*)
- EU-projektet POSEIDON arbejder med udvikling af renseteknikker, der sandsynligvis kan øge fjernelsen af hormonforstyrrende stoffer (*103*).

Nøgleresultaterne i det australske projekt har indtil i dag været en negligeabel fjernelse (< 10 %) af østron ved brug af jernkloridkoagulation og meget høj fjernelse (> 90 %) med aktivt kul. Magnetisk ionbytning har givet anledning til mellem 40 og 79 % fjernelse. Nanofiltrering viste en initial tilbageholdelse på 70-95 %, men for de fleste membraner faldt denne retention signifikant efter

den indledende filtreringsperiode. For nogle omvendt osmose-membraner svarede retentionseffektiviteten til det, der var opnået ved nanofiltrering, mens andre viste en meget høj stabil tilbageholdelse af stofferne. Mikrofiltrering viste ligeledes fuldstændig tilbageholdelse af stofferne i den initiale fase, hvorefter stoftilbageholdelsen som forventet faldt. De opnåede resultater bliver løbende efterprøvet i storskalasystemer (*102*).

Anvendeligheden af ozon og UV til behandling af udløbsvand fra renseanlæg undersøges i EU-projektet POSEIDON. De gennemførte undersøgelser har vist, at en initial ozonbehandling forbedrer betingelserne for en efterfølgende behandling med UV-lys. Ozonbehandlingen bevirkede, at absorptionskoefficienterne aftog, hvilket i sidste instans forbedrede UV-transmissionen fra 59 % til 84 %. De første resultater fra et pilotskalaforsøg viser en positiv behandlingseffekt i forhold til hormonforstyrrende stoffer. Driftsomkostningerne for ozonproduktion blev estimeret til mellem 0,90 and 1,60 EURO pr. kg ozon afhængig af energipriserne og systemets kapacitet (*103*).

Det kan konkluderes, at der er behov for mere viden om østrogeners og xenoøstrogeners skæbne i renseanlæg. Undersøgelser af høj kvalitet bør foretages af renseanlægs indløb, udløb og interne strømme med anvendelse af standardiserede prøveudtagningsmetoder og validerede analytiske metoder. Undersøgelserne skal kædes sammen med en omfattende monitering af renseanlæggenes generelle ydeevne og driftsforhold f.eks. belastningsgrad, næringssaltsfjernelse, hydrauliske opholdstid og slamaldre, som allerede foreslået af Johnson et al. (*95*). Undersøgelserne bør omfatte renseanlæg med forskellige typer af behandlingsprocesser med det formål at forstå betydningen af forskelle i procesdesign og driftskriterier. Moniteringen af fuldskalaanlæg bør ledsages af undersøgelser i pilot- og laboratorieskala.

Potentielle ikke-spildevandsrelaterede kilder til østrogen aktivitet i det vandige miljø
Ud over spildevand bør andre kilder til østrogener i det vandige miljø også komme under betragtning. To potentielle kilder er anvendelsen af gylle fra husdyrhold og af slam fra renseanlæg som gødning på marker. Der er p.t. dog utilstrækkelig viden om udledning af østrogener med drænvand til at vurdere, om disse er betydningsfulde kilder til østrogen aktivitet til overfladevand.

3 Reproductive disturbances in feral and caged fish from sewage effluent affected aquatic environments

3.1 The freshwater environment

3.1.1 Feral fish

3.1.1.1 Effects in male fish

The current concern about estrogens in the aquatic environment and their endocrine disrupting effects on fish has been raised by observations of feminised male fish made in England during the early and mid 90'ies (*1;104;105*). These observations supported the estrogen hypothesis put forward in 1993 by Skakkebæk and Sharpe which proposed that exposure of males (animals and humans) in a critical period of their life time to female hormones estrogens or to chemicals acting like estrogens (called xenoestrogens) can disrupt the normal development and impair the normal function of the male reproductive system (*106*).
Roach (*Rutilus rutilus*) collected from eight rivers throughout England downstream of discharges from sewage treatment plants (STP) were found to have a high frequency of intersex – an abnormal occurrence of hermaphoditism (*1*). Intersex was found in 16-100 % of male fish compared to 4-18 % intersex in males from control sites. In four sewage effluent receiving rivers the intersex frequency was above 50 % and in two of these, all examined males were showing feminisation. The intersex condition was characterised by a presence of both early and developed stages of egg cells (oocytes), and the presence of the female duct, the ovarian cavity, in the testes of presumptive male fish. The ovarian cavity is the structure within the ovary to which mature eggs are released before being passed on to the oviduct. The severity of the condition varied among individuals from only a few primary oocytes interspersed among otherwise normal testicular tissue to testes where more than 50 % of the tissue was female. In some cases the male sperm duct was further missing. The study showed a correlation between the degree of intersex and the concentration of sewage effluent in the river indicating that compounds in the sewage effluent were responsible for inducing the intersex condition. Observations of concomitantly high levels of the female egg yolk protein vitellogenin (vitellogenin) in sewage effluent exposed male fish indicated exposure to estrogens and/or xenoestrogens which has been verified by cage experiments and chemical analyses (*25;104;107*) (see section 3.1.2.1 and chapter 4).

Synthesis of vitellogenin by male or immature fish is an internationally used marker for estrogenic exposure. Vitellogenin is normally produced by female fish during their reproductive cycle. It is induced in the liver by the endogenous estradiol production of the female, excreted to the blood stream and incorporated into the developing oocytes in which it is degraded to yolk proteins. These act as nutrition for the developing larvae (*108*). Males and

immature fish normally contain no or very little vitellogenin in plasma possibly due to very low circulating levels of estradiol. If male fish are exposed to (xeno-)estrogens this will, however, induce the synthesis of vitellogenin which in severe cases can reach levels as high as or higher than found in sexually mature females.

A second species of cyprinid fish, the gudgeon (*Gobio gocio*) has also been demonstrated to show intersex in English rivers (*15*). The frequency was not as high as demonstrated among roach from the same rivers. This is despite the fact that the gudgeon is a bottom living fish which could be expected to be exposed to compounds both in the water phase and in the sediment. The highest frequency of intersex found in this species was 15 %. This was found at the same river site from which 100 % intersex was seen among roach. However, in line with the high frequency of disruption in roach, the most severe cases of intersex among gudgeon were found among fish from this site and the male fish also had elevated levels of vitellogenin. As for roach, intersex in gudgeon seems to occur to some extent naturally since it was found at a background frequency of 6 % at control sites without sewage effluent discharges. In general, however, this species seems to be less sensitive compared to roach towards endocrine disruption.

The observations of feminised feral fish from English rivers have led to studies of roach populations in both Denmark and France and of populations of other fish species in other European countries and USA.
In Denmark intersex has been found in 7, 11 and 27 % of male roach collected in 1999 from three sewage effluent receiving streams in Aarhus County. The highest frequency of intersex occurred in the stream, Kristrup Landkanal which receives sewage from the city of Randers and which all year round consists of more than 97 % sewage effluent. Among the roach populations from two clean lakes with no known effluent discharge intersex occurred in 4.5 -5 % of male fish (*2*). A tendency to a higher degree or extent of feminisation was found in intersex individuals from the three sewage effluent affected streams, but only significantly at one site, compared to the extent in intersex fish from the two control sites. In general, a milder degree of feminisation was found in the examined Danish roach populations compared to that observed in English roach. Occurrence of the female ovarian cavity was not observed and only primary growth stages of oocytes were seen from few to a maximum of 200 per testes section.

In accordance with the Danish study, a study of roach from 4 rivers in the north eastern part of France concluded a lower incidence of intersex compared to English roach. An intersex frequency of 0-40 % and 2-60 oocytes per histological testes section were found (*13*).
In France estrogenic effects have also been observed in a preliminary study of another fish species the chub (*Leuciscus cephalus*) from one river (*7*). Male fish from a polluted river stretch of Moselle had higher levels of vitellogenin compared to fish from a control river and 50 % of the males had necrotic sperm cells whereas this was not seen in males from the unpolluted river. This study is, however, a study based on relatively few fish from each site.

Brown trout (*Salmo trutta*) is another fresh water species which has been studied in Denmark with regard to endocrine disruption (*2*). In one stream with low sewage effluent load, Voel Brook, an increased level of vitellogenin was found among males. Fortyfour % of these males also had testes with a

high degree of vacuolation[4] and only a presence of the earliest sperm cell stage, spermatogonia. This was also found in 5 % of fish from a clean control site and in approximately 2 % of fish from two other sewage effluent receiving brooks. The high levels of vitellogenin in males from Voel show that the fish have been exposed to estrogens and/or xenoestrogens. Whether the different testes structure compared to most control fish also was a consequence of hormonal exposure or is due to other factors such as a different age structure among fish from Voel or exposure to other chemicals is not known. Vacuolation in sertoli cells in the brown trout can be the result of the reabsorption of sperm cells which have not been spawned in the previous spawning cycle or absorption of degenerated sperm cell stages from the present reproductive cycle (*109;110*).

In Spain, male carp (*Cyprinus carpio*) has been examined for feminising effects in two rivers, Anoia and Cardener, with high and low to moderate presence of estrogens, respectively (*8;14*). Males from both rivers showed presence of vitellogenin in plasma in approximately 30 % of the examined fish. In accordance with the higher estrogenic load in the Anoia river, male carp from this river showed a higher plasmatic vitellogenin concentration than males from Cardener River. Intersex fish with simultaneous presence of testicular and ovarian tissue were further observed among fish from River Anoia. In this case the presence of high vitellogenin levels in the male fish was not consequently indicative of gonadal effects since intersex fish both were found to have vitellogenin levels higher than females and levels not significantly different from control males.

A large national survey in the Netherlands of occurrence and effect of estrogens and xenoestrogens in the aquatic environment has examined the reproductive health of approximately 800 bream (*Abramis brama*) (*6*). High levels of vitellogenin were detected in male bream from several freshwater sites including the rivers Rhine and Meuse and in three major river sedimentation areas. The highest vitellogenin concentrations were found among males from a small stream, Drommel, from which the largest percentage of intersex was also recorded. Fortyseven % male fish collected in spring and 33 % of male fish collected in fall had oocytes in the testes. Lower frequencies of intersex (4 % and 9 % respectively) were found at two other sites. Initial results from other smaller Dutch waters, which have not been published yet, have indicated that in one other stream, equally high levels of vitellogenin and intersex in male bream as in the Drommel were found. Generally, estrogenic effects appeared at half the selected locations (*6*).

The bream population in the river Elbe in Germany has also been examined for endocrine disruption at nine different sites from Hamburg to the Czech border (*5*). Weak estrogenic effects were seen along the entire length of the river since higher levels of vitellogenin in male bream were found at all sites compared to levels in males from a control lake with no domestic or industrial effluent input. The strongest estrogenic influence, detected as the highest vitellogenin induction (20-100 times the level in controls), was found at locations characterised by high levels of effluent discharge to the river (discharges from STPs with 825,000 and 1,534,000 population equivalents (P.E.s)[5], respectively). Conversely, no marked vitellogenin induction was

[4] : An empty vesicle within a cell – in this case apparently in sertoli cells which functions among other things as nursing cells for the development of germ cells (*110*)
[5] : P:E.: One population equivalent (P.E.) is the amount of organic biodegradable load which has a biochemical demand (BOD5) of 60 g per day (*1*).

found downstream of a very large STP discharge (2,000,000 PE at Hamburg) probably reflecting the importance of the greater dilution and better treatment of the sewage at this site compared to the above mentioned. Suppression of reproductive function was also assessed by altered steroid hormone status (lowered 17β-estradiol in females and lowered 11-ketotestosterone in males) and lowered gonadosomatic index (GSI)[6] in both sexes. Low incidences of intersex were found at all sites along the Elbe from 2-6 % compared to 0.5 % fish with ovotestis in the control lake. Mostly, mild degrees of intersex were observed but cases where more than 45 % of the testis was ovarian were also found. In general it was concluded that disruption of the endocrine system of the bream population was observed but only the higher levels of vitellogenin were ascribed to estrogenic exposure whereas other effects might have happened through multiple mechanisms also involving other groups of chemicals such as polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) also known to exist in higher levels in the Elbe. Levels of estrogens were not measured (*5*).

Outside Europe intersex has been detected in 29 % of shovelnose turgeon (*Scaphirhynchus platyorynchus*) in USA from a stretch of the Mississippi River which is rich in contaminants (*9*). Other studies of carp (*Cyprinus carpio*) and walleye (*Stizostedion vitreum*) have demonstrated higher vitellogenin levels in males collected near discharges from STPs (*11*). In both species lower levels of serum testosterone were also detected in male fish. In walleye a delay in spermatogenesis relative to fish from control sites was observed and there were indications that viable sperm was not being produced in males from the STP site. Again another American study has shown that there in a population of chinook salmon (*Oncorhynchus tshawytcha*) were 84 % of phenotypical[7] females (having both female external secondary sexual characters and ovaries) which were genotypical[8] males. These fish were therefore apparently completely sex reversed males (*111*). A connection to a chemical/compound release has not been found.

3.1.1.2 Effects in female fish

Due to their low endogenous levels of estrogens, male fish are considered to be the most sensitive sex towards reproductive disrupting effects caused by estrogenic compounds in sewage effluent. There does, however, exist some observations indicating that endocrine disruption of female feral fish has also taken place.

New results on English roach from two of the most affected rivers in which males had developed intersex have also demonstrated a higher incidence of oocyte atresia or degeneration in the sewage effluent-exposed females compared to control females. Sex hormone levels were also disturbed since the estradiol concentration was only half that measured in control fish illustrating that the female reproductive system was also affected (*60*).

Another report on female reproductive effects come from a study in Sweden reported in 2001, which described endocrine disruption in fish from a lake which was receiving leakage water from a nearby refuse dump(*112*). The situation is therefore not entirely comparable to the exposure cases with STP,

[6] : GSI: Weight of gonad relative to total body weight
[7] : Phentypical: the observable constitution of an organism
[8] : Genotypical: the genetic constitution of an organism

but included since strong indications of endocrine disruption were seen. Effects were observed in both males and females but with the most pronounced effects in females. A large percentage (75 %) of the females was found to be sexually immature compared to fish from a clean reference lake. They showed an 80 % reduction in the gonadosomatic index (GSI) which shows a marked reduction in gonadal size. Among the sexually mature females a lower aromatase[9] activity in the brain and altered steroid levels in plasma were detected. Testosterone levels were lower in fish from the leakage water affected lake and a concomitant tendency to a lower level of plasma estradiol was observed. The authors suggested that the reduced level of estradiol might be a result of the lower brain aromatase activity and that the reduced estradiol production could have lowered the production of vitellogenin and therefore inhibited the development of oocytes in the females. A reduced GSI (23 %) and reduced plasma testosterone levels were also observed among female roach from the same lake. In both perch (*Perca fluvalis*) and roach reductions in GSI were also seen in male fish with a 36 % reduction in male roach.

Changed steroid levels have also been reported in female walleye from a sewage effluent affected site in the USA (*11*).

3.1.2 Cage experiments and other controlled sewage effluent exposure studies

A number of caging experiments where fish have been placed in streams in close proximity to the STP outlets and experiments where fish have been exposed to various controlled dilutions of sewage effluent have verified the estrogenic and feminising capacity of sewage effluent from several STPs on male fish. Dose-response relationships among effluent dilution and feminising effects have been demonstrated (*4;24;107;113;114*) along with consequences of exposure time (*24*).

3.1.2.1 Cage experiments

A series of cage experiments in England where male rainbow trout (*Oncorhynchus mykiss*) were placed in cages for three weeks in vicinity of discharges from STPs were the first to demonstrate the estrogenic nature of UK sewage effluent being discharged to English rivers through observations of up to a 10,000-fold induction of vitellogenin in male fish (*104;105*). The survey of six rivers demonstrated induction of vitellogenin in caged fish in five of these rivers. In one river the estrogenicity was shown to persist as far as 5 km from the discharge point but was in the other rivers (in a three week trial) only observed in immediate vicinity of the STPs (*107*). Fractionation of the sewage effluent and chemical analysis of the water have subsequently shown that the compounds responsible for the observed feminisation in most cases primarily were natural and synthetic estrogens, 17β-estradiol, oestrone and ethinylestradiol (*25*). In the case of one river, however, outlets of alkylphenolic chemicals with industrial sewage from wool scouring and other textile industries (*34*) were assessed to contribute to the majority of the estrogenic activity.

Estrogens from humans are excreted in a hormonally inactive form as steroid glucoronides and sulphates primarily via urine. The steroid conjugation takes

[9] Aromatase: The steroidogenic enzyme which converts aromatisable androgens to estrogen.

place in the liver and is a normal metabolic pathway used to facilitate the excretion of compounds from the body (*115*). In humans estrogens are primarily excreted as estrogen glucuronide conjugates. Women may excrete around 2.4 µg of estradiol, 4.6 µg of estriol and 7 µg of estrone daily via urine. Smaller quantities are also excreted with faeces (in (*116*)). (For more information regarding women as a source of estrogens see section 10). The microbial activity in the sewage treatment works and possibly also in the sewage before it reaches the treatment works (*117*) results, however, in a deconjugation of the steroid thereby restoring the estrogenicity of the steroid components of the sewage effluent (*118*).

Cage experiments using juvenile rainbow trout have also demonstrated the estrogenic nature of Swedish sewage effluent from a relatively small STP which mainly receives domestic sewage (*3*). The STP had chemical and biological treatment steps but no anaerobic denitrification step and processed sewage effluent from a smaller number of people (3500). Still, a very high induction of vitellogenin was found in male fish after an exposure period of two weeks in the stream. Both estradiol, estrone, ethinylestradiol, nonylphenol and bisphenol A could be detected in the bile of the fish. Chemical analyses of the stream water showed high levels of all three estrogens but with ethinylestradiol at a 45 times higher concentration (4.5 ng/l) than the one experimentally set as lowest concentration which will induce estrogenic effects in fish (*119*). Ethinylestradiol was therefore considered to be responsible for most of the estrogenicity of the examined effluent.

Very high levels of EE2 detected in a final STP effluent in Texas were also considered the likely cause of elevated vitellogenin levels, reduced GSI and reduced secondary sexual characteristics in fathead minnows (*Pimephales promelas*) kept for three weeks in cages in a treatment wetland close to the outlet of effluent (*120*). EE2 was measured in concentrations as high as 200-900 ng/l. No information were given regarding the treatment steps of the STP from which the effluent was coming.
On the contrary, no effects on the same parameters in a similar study of fathead minnow were observed when caged fish were exposed *in situ* at riverine sites to representative effluents from central Michigan sewage treatment plants (*121*). All plants were using at least secondary treatment technologies and at some sites tertiary treatment such as sand or charcoal filters were also applied. Chemical analysis of possible estrogenic compounds in the sewage effluent or surface water was not performed.

Different sensitivities of different fish species have to be taken into consideration when using cage studies to assess or confirm the estrogenic capacity of sewage effluent. In the dutch study mentioned above where a high degree of feminisation was observed in wild bream from the small stream, Drommel, cage experiments in the same stream with either carp or rainbow trout showed high vitellogenin induction in rainbow trout but not in carp when male fish were placed in cages for 21 days (*6*). The results from the cage experiments with rainbow trout did, however, confirm that the feminisation of feral bream could be ascribed to sewage effluent exposure. This was confirmed by dose-response related vitellogenin induction in rainbow trout (but again not in carp) with a threshold concentration of 25-50 % sewage from two of the sites with feminisation of wild bream.

The Danish studies which have examined the estrogenicity of sewage by caged fish have in general found no or a relatively weak induction of

vitellogenin in the sewage exposed fish. A weak vitellogenin induction has been found after 6 weeks among rainbow trout placed near the outlet of sewage from the treatment plant, Ejby Mølle, Odense (*122*) and in flounder exposed for three weeks to sewage effluent from Avedøre STP (*123*). In the last mentioned study no induction was, however, not seen in two other species, rainbow trout and eel (*Anguilla anguilla*). A study in which rainbow trout were placed in cages for 9 weeks in a highly sewage effluent receiving brook, Kristrup Landkanal and in the brook, Egå for 13 weeks also did not cause induction of vitellogenin (*124*). This is despite the observation of relatively high frequencies of intersex among roach from Kristrup Landkanal. The explanation for this discrepancy might be differences in exposure time for the two species. Further, for the Atlantic croaker (*Micropogonias undulatus*), it has been demonstrated that the estrogen receptor in the testis is saturated at four times lower concentrations of estrogens and a number of estrogenic compounds compared to the estrogen receptor in the liver, in which the vitellogenin synthesis is induced. Alterations in the gonads such as intersex or sex reversal might therefore be more sensitive indicators of an estrogen effect than vitellogenin.

3.1.2.2 Other controlled experiments with graded concentrations of sewage effluent

In line with the last study just mentioned, a number of controlled studies with graded concentration of sewage effluent have further demonstrated a dose-response relationship between vitellogenin induction or intersex and the percentage of sewage effluent verifying again the estrogenicity of sewage effluent from several European countries and adding proof to the sewage effluent as the source of compounds causing the feminisations of wild fish.

Sewage effluent from a municipal sewage plant which receives domestic sewage from Oslo was estrogenic to rainbow trout at concentration of 2.5, 10 and 20 % sewage when exposing the fish for 3 weeks (*4*). In the same study effluent from an oil refinery treatment works was estrogenic to rainbow trout. A Swedish study has also demonstrated estrogenicity of 50 % effluent from a pulp mill (*125*) by vitellogenin induction in zebrafish (*Danio rerio*). Concerns about endocrine disruption by effluent from pulp mills have mainly been in the direction of masculinising effects, which has been seen in a number of cases (*115;126*). Both estrogenicity and androgenicity of the effluent were observed in the Swedish study since exposure to the pulp mill effluent besides inducing vitellogenin also resulted in a higher proportion of males. This indicates the presence of both estrogenic and androgenic compounds in the water.

Increasing levels of vitellogenin in male rainbow trout exposed for six months to increasing levels of effluent (10, 20, 30, 40 %) from the Berlin-Ruhleben sewage plant in Germany have also been found(*114;127*). Ethinylestradiol, 17ß-estradiol and estrone were measured at concentrations of <0.2-3 ng/l, <0.5-1.5 ng/l and 0.5-1 ng/l, respectively in the effluent which, compared to the measured concentration of xenoestrogens i.e. alkylphenols and bisphenol A, were closer to the concentrations known to exert effects on fish (see chapter 6) (*127*).

In roach, it has further been shown that vitellogenin synthesis induced by sewage effluent besides being dose dependent also depends on the exposure period (*24*). The percentage of the sewage effluent which was able to induce

vitellogenin in mature adult roach fell from 37.9 to 9.4 % sewage effluent by prolonging the exposure period from 1 to 4 months. In the sewage effluent used in this study the levels of estrogens were 1.7-3.4 ng/l ethinylestradiol, 4-88 ng/l 17ß-estradiol and 15-220 ng/l estrone also illustrating the high levels of all three hormones relative to their estrogenic potential (see chapter 6) but also the great fluctuation in hormone levels over time (8 months).
Sewage from the same STP could in another study induce a dose-response dependent development of intersex in roach when exposing juvenile fish from 50 to 200 days after hatch. The fish developed the female duct called the ovarian cavity also detected in wild roach (*1*) at a threshold concentration of 23.6 % sewage. Exposure to 100 % effluent resulted in 100 % occurrence of intersex in males. This feature seemed to be irreversible since depuration of the effluent exposed fish in clean water for 150 days did not result in any alteration in the duct formation. No induction of oocytes was induced in the testes of any male fish in the present study and therefore the causality of the oocytes in the testes in wild roach still needs complete elucidation. Since other studies have demonstrated induction of ovotestes with (xeno-)estrogens (*53;128*), the lack of oocyte induction in males in the study with juvenile roach might be because the exposure period did not cover the sensitive window/critical period for development of this feature (*113*).

3.2 The marine environment

Marine organisms have been expected to be less affected by endocrine disruption from estrogenic discharges to the marine environment compared to freshwater fauna due to the usually high degree of dilution of sewage effluent in estuaries and open waters (*18*). A number of surveys have, however, demonstrated feminisation of some marine species, predominantly in flounder (*Platichthys flesus*) (*16-22*). The flounder has been chosen as a suitable marine test monitoring species because it lives in close contact with the sediment in which it buries itself and it lives most of the time in the estuaries migrating only to sea for breeding (*19*). One disadvantage with this species is, however, that controlled laboratory studies using water exposure have demonstrated the flounder to be less sensitive towards estrogens compared to for instance the rainbow trout (*17*). Still, elevated levels of vitellogenin has been detected in flounder from several industrialised estuaries in England (especially in Tyne, Tees and Mersey) compared to reference sites in a number of studies conducted over a period of 6 years (*16-18;21;22*). The routes by which flounder is exposed to environmental estrogens are not fully understood. Exposure through the food chain might contribute to the observed effects in flounder since experiments where flounders were fed mussels held in the Tees estuary for 3 months showed a 10-fold increase in plasma vitellogenin (*18*). Exposure of male flounder to water and sediment in cages at two sites of the Tees and Tyne for three weeks did not induce vitellogenin production (*129*). Occasionally high vitellogenin levels have also been detected among males collected offshore. The reason for this is unclear but might rather be due to migration of males from the more polluted estuarine areas than caused by local pollution offshore (*16*). Similar observations of single very high levels of vitellogenin among male flounder from several near cost areas in Denmark have been made (*130*). How these results should be interpreted is also very uncertain and a need for more knowledge on seasonal and age related vitellogenin levels in this species is needed.

Intersex has also been detected among male flounder in the English surveys. Depending on the year of sampling, intersex has been found in between 7 and

20 % of male fish from the sites where also high vitellogenin induction was found among males. Other gonad changes which have been reported from flounder are abnormal testes morphology such as disorganised lobules[10], vacuolation, thickening of connective tissue and lack of germ cells pointing toward an inhibited spermatogenesis (*22*). These changes were detected in a higher proportion (53-67 %, depending on the time of the year) in the fish from the polluted Tyne estuary compared to fish from a reference site (maximum 12 %). A higher proportion of degenerating oocytes were seen among females from Tyne in the same study.

In a Dutch study of offshore, estuarine and inland locations including 1400 flounders, levels of vitellogenin were found to be low among males at most investigated sites (*6*) except at two sites in the North Sea Canal, an industrialised port zone which receives effluent from sewage treatment works. Here moderately elevated levels of vitellogenin were found among the examined males in a fall but not a spring sampling. None of the captured males had oocytes in their testes. Based on these results it was therefore concluded that estrogenic effects in the marine waters of the Netherlands were few compared to the situation reported from the English and Welch Coast.

Both a higher level of vitellogenin and intersex were found in male flounder from the Tokyo Bay which receives large amounts of industrial and domestic sewage effluent. Oocytes were found in testes of 15 % of the males compared to none in the control population (*12*).

As mentioned, studies of feminising effects on marine fish species have mainly concentrated on the flounder but a few results on other species have been reported. A small scale study of the eelpout (*Zoarches viviparous*) from English estuaries has shown that up to 85 % of the males caught at particular stations contained vitellogenin mRNA in the liver compaired to none at a reference site. Gonads were normal among the control individuals whereas intersex was found in up to 17 % of male from polluted estuaries (*18*).

Two species of sand gobies (*Pomatoschistus minutus* and *P. lozanoi*) have recently also been proposed as suitable monitoring organisms for endocrine disruption related to estrogen exposure (*18;23*). A phenomenon called Morphologically Intermediate Papilla Syndrome (MIPS) has been found in 33-75 % of males from four estuarine sites with known estrogenic contamination compared to a 0-15 % prevalence of the condition among males from the control and other estuarine sites. MIPS is a condition in which the male urogenital papilla (a secondary sexual organ used for depositing sperm) has developed villi at the papilla tips which resembles the villi on the female papilla used for oviposition. This is a condition which has been demonstrated to be inducible by controlled exposure to estradiol (*18*).

[10] : The testis contains numurous socalled testicular tubules with a central lumen. The development of sperm cells takes place along the entire length of the tubule periphery. At maturity, spermatozoa are released to the tubule lumen and transported to the sperm duct (*269*).

4 Quantification of the estrogenicity of sewage effluent using *in vitro* assays and the TIE approach.

The observations of feminisation of fish exposed to sewage effluent, indicating that the effluent contains estrogenic compounds, have led to attempts of quantifying the estrogenic activity of the effluent. Fractionation techniques followed by *in vitro* assays have been used to quantify the estrogenic activity of the sewage effluent. The fractions responsible for the estrogenic activities in the *in vitro* assays have in some cases further been subjected to chemical analyses leading to identification of the active chemicals (*25*) - a procedure called toxicology identification evaluation (TIE).

The following chapter will summarise the results made on estrogenicity of sewage effluent with numerous *in vitro* assays and compare these with the actual concentrations measured in the sewage effluent where these have been made. Additional results on estrogen concentrations from chemical analysis of sewage effluent and surface water will be presented in chapter 5.

The additive behaviour of the estrogenic activity of single substances in a mixture has been demonstrated and is the basis for quantitatively assessing the total contents of estrogenic substances in an environmental sample by the use of *in vitro* assays. The total estrogenicity is then compared to the magnitude of response elicited by 17ß-estradiol and described as estrogen equivalents (*30*).

A number of cell based *in vitro* assays have been applied which uses cells transfected with an estrogen receptor controlled reporter gene (*30;30;32*). Among these is the YES assay using yeast cells transfected with the human estrogen receptor (ER) and the reporter gene β-galactosidase (*25*). Other assays use transfected human breast cancer cells transfected i.e. with luciferase in the ER-CALUX assay (*32*). The human breast cancer cells, of which different strains have been used, already contain the human estrogen receptor. The E-screen, in which proliferation of human breast cancer cells (MCF-7) as a response to estrogen is measured, has also been used to determine the estrogenicity of sewage effluent and surface water (*26-28;131*).
One drawback which has to be considered when using these types of assays is, however, that antiestrogens which might also be present in the water samples can bind to the estrogen receptor and counteract the estrogenic response. An underestimation of the actual estrogenic potential of the water source might therefore take place. This has been demonstrated earlier (*132*). Further, several *in vitro* assays have found approximately the same estrogenicity of 17ß-estradiol and ethinylestradiol while the ethinylestradiol *in vivo* has been found to have an approximately 10 times higher estrogenicitet than estradiol. This might also lead to an underestimation of the actual feminising potential of a water source.

Körner et al. 1999, 2000, 2001 have used the E-screen to assess the estrogenicity of sewage effluent and sludge from German sewage treatment plants (*26-28*). Analysis of sewage from five different municipal sewage

treatment plants in South Germany detected 2-25 ng/l estrogen equivalents (EEQs). The plants all had mechanical purification devices (primary clarification), activated sludge treatment, biological nitrate removal (nitrate/denitrification) and final settlement tanks. In another study EEQs of between 0.2 and 7.8 ng/l EEQ were detected (median 1.6 ng/l) in effluents from 16 municipal and two industrial STPs in the state of Baden-Wüttemberg, Germany (*26*). This study indicated rather constant inputs of estrogenic substances via STP effluent to the rivers. This is not in agreement with an English study which demonstrated very variable estrogen levels in sewage effluent analysed over a period of 8 months (*133*).
Effluent from a modern municipal STP in Germany with a technical standard reported to be very high still contained 6 ng/l EEQ (*28*). The municipal STP processed sewage from 200,000 inhabitants and was reported to have a capacity of 350,000 population equivalents (P.E.)[11]. 60 % of the wastewater was of domestic origin and 40 % of industrial or other origin. Chemical analysis of the effluent water detected 4-*tert*-octylphenol (4-*t*OP), 4-nonylphenol (NP) and bisphenol A in the effluent at concentrations from 0.16 to 0.36 µg/l and the contribution to the total estrogenicity of the sewage effluent coming from these compounds was assessed to 0.7-4.3 %. This indicated that most of the estrogenicity of the effluent was due to the presence of natural and synthetic estrogens (*28*). Since the effluent was diluted to 50 % in the Danube River *in vivo* estrogenicity on the river fauna was not expected by the authors.
Estrogenicity suspected to impact fish has, however, been demonstrated for another large German river, the Rhine, despite a 10-100 times lower YES estrogenicity of the river water compared to the effluent. Chemical analysis detected estradiol at relevant concentrations of 3.9 ng/l in the River Rhine along with some phytosteroids, and estradiol was thought to explain part of the *in vitro* and *in vivo* induction of vitellogenin which was obtained by exposure of rainbow trout hepatocytes and male rainbow trout, respectively to river water (*134*).

Natural and synthetic estrogens have also been considered responsible for the observed estrogenicity of sewage effluent in other studies. Sewage effluent from seven STPs which discharged into English Rivers were demonstrated to contain three fractions with estrogenic activity when these were tested with the YES assay (*25*). Chemical analysis isolated 17β-estradiol, estrone and ethinylestradiol from these fractions. Estradiol and estrone were detected in all samples in concentrations of 1- 48 ng/l and 1 – 76 ng/l, respectively while ethinylestradiol was detected in three effluents at concentrations of 0.2 – 7.0 ng/l. These levels have led to the conclusion that most effects on freshwater feral fish observed in English rivers are a result of natural estrogens excreted from women. The effluent tested in this study contained little or no agricultural input and all natural estrogen input was believed to be of human origin.
The feminising effects which have been found in English estuaries in feral flounder also seem to be related to the presence of the natural hormone estradiol in the effluent (*129*). This was the major component causing 84-90 % of the estrogenicity in a YES assay although the magnitude of the response of a maximum of 24 ng/l EEQ was less than observed in freshwater environments in England. The sediment pore water was also found to have an

[11] P:E.: One population equivalent (P.E.) is the amount of organic biodegradable load which has a biochemical demand (BOD5) of 60 g per day (*1*).

estrogenic effect equivalent of 7 ng E2/l but the responsible compounds were not identified.

The estrogen equivalent levels were also found to be higher in Dutch fresh water compartments and biota compared to coastal and marine environments (*116*)

The estrogenic activity of 2.2 – 12 ng EEQs/l which has been detected by *in vitro* assays at Meiliang Bay of Taihu Lake - the third largest lake of China – has also been ascribed mainly to estradiol and ethinylestradiol (*29*). This lake has been described as one of the most polluted water bodies of China and both municipal and industrial sewage from a city of 4 mio inhabitants are discharged into the bay. Estradiol at concentrations from 1.6 – 15.5 ng/l and concentration of ethinylestradiol from 5.7 – 30.8 ng/l have been measured.

In effluents from three municipal wastewater treatment plants in Michigan, four point source locations and five locations in Lake Mead, EEQs of 1.90 – 14.9 ng/l, 3.64 – 5.30 ng/l and 1.08 – 10.9 ng/l, respectively have been found by Snyder (*30*). Estradiol and ethinylestradiol accounted for 88 to 99.5 % of the total estrogen equivalents in the water samples and although alkylphenols were detected in up to 37 µg/l water they were only assessed to contribute to less than 0.5 % of the total EEQ.

In a Korean river total estrogenic activity was 0.5 – 7.4 ng EEQ/l in water downstream of sewage discharge with a sediment activity of 3.39 – 10.70 ng EEQ/g (*131*). In a Japanese river EEQs of 3.5 ng/l have been reported (*135*). Effluent from two French STPs which received mainly domestic wastewater (80,000 and 300,000 P.E.s, respectively) had a total estrogenic activity of 1.36 - > 8.17 ng EEQ/l, and estrogenic activity of 0.27-1.36 EEQ/l was also detected in receiving river water (*136*). In a South African study of 25 selected water and sediment samples using the YES assay, estrogenic potencies ranged from below detection limit (0.027 ng/l) to 23.5 ng EEQ/l (*137*) and from below detection limit (0.020 ng/g) to 13,9 ng/g for the sediment samples.

The last results which will be mentioned from studies assessing the *in vitro* estrogenicity are on sewage and surface water in the Netherlands and Belgium. The Dutch national survey recently reported 0.03-16.01 ng EEQ/l in sewage but only 0.07-0.47 ng EEQ/l in river water. More than 80 % of the estrogenic activity of the effluent could, however, not be explained by chemical analysis of the known (xeno)-estrogens (*6;32*).
A study of the estrogenic activity of Flemish rivers and effluent surprisingly found the highest estrogenic potency in the surface water samples compared to the effluent (*33*). The estrogenic potency of the water samples ranged from below detection (~ 2.75 ng EEQ/l) to 81 ng EEQ/l. More than 10 ng EEQ/l were found in 7 of 16 samples.

As described above the use of *in vitro* assays has demonstrated estrogenicity of sewage in numerous countries and in a large number of these studies the major part of the *in vitro* estrogenicity was assigned to the presence of natural and synthetic estrogens in the samples. Therefore the following sections describing environmental concentrations in relation to known dose-response relationships regarding endocrine disrupting effects will concentrate on the natural estrogens 17β-estradiol, estrone and estriol, the synthetic estrogen

ethinylestradiol and among xenoestrogens the more potent alkylphenols and bisphenol A.

5 Occurrence of estrogens and xenoestrogens in sewage effluent and occurrence and fate in the aquatic environment

The present section will describe the occurrence of estrogens and xenoestrogens in the sewage effluent and what is known about their occurrence and fate when they reach the aquatic environment. Some information on the actual concentrations in both final effluent and surface water has already been given in chapter 4 in relation to the *in vitro* estrogenicity of the water samples. Table 5.1.
and 5.2 summarise additional concentrations which have been reported for natural and synthetic estrogens in effluent and surface water, respectively while concentrations for the alkylphenolic compounds and bisphenol A are given in Table 5.3-5.6. Since a range of studies as shown in the previous section has pinpointed natural and synthetic estrogens to be the primary causes of the observed estrogenic activity of sewage effluent in many countries these will receive the largest attention in the following section compared to alkylphenols and bisphenol A.
The fate of the estrogens in the sewage treatment works and the influence of different treatment processes on the removal rate of these compounds from the sewage will be covered in Chapter 11 and 12.

An important thing which has to be remembered whenever concentrations of estrogens are mentioned in the present report is, however, that it seldom is specified whether the chemical analysis measures only free or both free and conjugated estrogens. There must probably also be expected to be larger uncertainties related to measurements of estrogen concentrations in influent than in effluent due primarily to interference from large contents of organic matter (matrix effect).Further, the detection limits of the analyses, especially for ethinylestradiol, are close to and sometimes even above the concentrations which have been demonstrated to cause reproductive disturbances in fish.

5.1 Estrogens

5.1.1 Occurrence in sewage effluent

When comparing the estrogen concentrations in sewage effluent from numerous countries mentioned throughout this report it is apparent that the levels can vary a lot. Levels of < 0.1 – 88 ng/l 17ß-estradiol, < 0.1 – 220 ng/l estrone, and < 0.053 – 62 ng/l ethinylestradiol have been detected (Table 5.1. and Chapter 4). The typical range of the
steroids is in the range of 1-10 ng/l and 5-20 ng/l for estradiol and estrone, respectively, while ethinylestradiol often is below detection limits (0.1 –1 ng/l). When detected, it is mostly below 10 ng/l. Estrone is the most frequently detected of the natural estrogens and often detected in all samples which might reflect the relatively fast conversion of 17ß-estradiol to estrone (see

chapter 5.1.3.1). Estriol could be expected at higher concentrations than estrone since this is excreted in higher concentrations from women. The relatively few measurements which exist on the effluent concentrations of this steroid have detected it in the range of < 0.1 – 42 ng/l estriol. Due to the relatively sparse material on effluent concentrations of estriol it is not possible to say whether these are representative.

The steroid concentrations which have been measured in Danish sewage effluent lie within the typical range of the steroid estrogens which have been reported from other European countries.

Large differences in effluent concentrations from a single sewage treatment plant are often observed when concentrations are followed over time. This might be caused by different weather conditions (sunshine and dryness, dilution by rainfall, temperature) in the sampling period, differences in the residence time of the sewage, microbial activity and varying composition of the influent water (*138;139*). The differences in steroid hormone concentration between effluent from different treatment works will also depend on the type of treatment processes used by the plant. This will be discussed in Chapter 13.
Repetitive samplings of sewage effluent might therefore be necessary in order to get a true picture of the range of estrogen concentrations being released with sewage effluent.

It also has to be kept in mind when relating the estrogen concentrations in sewage effluent to known lowest observed effect concentrations (LOECs) and no observed effect concentration (NOECs) for endocrine disrupting effects on fish that the reported concentrations often are measured in 24 hour samplings which gives the average steroid concentration being released to the water phase. It is known, however, that there is en early morning surge of sewage into the STPs (*140*) and there might therefore be higher peak values released to the environment at certain hours of the day.

Table 5.1 Concentrations of natural and synthetic estrogens in sewage effluent

Country	Compound	Conc. (ng/l)[1]	LOD (ng/l)	Method	Comments	Ref.
Denmark	17β-estradiol estrone ethinylestradiol	< LOD – 2.5 < LOD – 6.1 < LOD – 4.7	1 1 1	GC-MS/MS	Measured in effluent from 8 STPs in Aarhus County (n = 3 for each). STPs all had mechanical and biological treatment steps with a nitrification step. Some had additional denitrification, filter and chemical steps. E2 detected in 2, E1 in 22 and EE2 in 2 of 24 samples.	(*2*)
Denmark	17β-estradiol estrone ethinylestradiol	< LOD < LOD – 5.2 < LOD	1 1 1	GC-MS/MS	Measured in effluent from seven low-technology sewage treatment works.	(*2*)
Denmark	17β-estradiol estrone ethinylestradiol	< LOD – 11 (< 1) < LOD – 63 (< 1) 4.9 - 7.0 (6.3)	1 1 1	GC-MS/MS	Detected in effluent from four STPs in Copenhagen	(*141*)
England	17β-estradiol estrone estriol ethinylestradiol	LOD – 0.9 LOD – 3.4 LOD – 0.2 < LOD	- - - -	GC-MS	Effluent from two STPs in North London	(*142*)
England	17β-estradiol estrone estriol ethinylestradiol	1.6 – 7.4 6.4 – 29 2.0 – 4.0 < LOD	- - - -	GC-MS	Three STP effluents. All used activated sludge processes.	(*139*)
England	17β-estradiol estrone ethinylestradiol	< LOD – 7.97 0.8 – 2.45 0.56	1 1 1	GC-MS/MS	Three STPs; two with activated sludge treatment and one with a percolating filter process.	(*35*)
England	17β-estradiol estrone ethinylestradiol	5 18 < 0.1	- - -	GC-MS	One STP. Mean concentrations	(*34*)
Germany	17β-estradiol estrone ethinylestradiol	0.15 – 5.2 (0.4) 0.35 – 18 (1.5) 0.1 – 8.9 (0.7)	0.15 0.1 0.1	HRGC-(NCI)-MS	Results from three municipal STPs sampled over 5 months (total n = 16). The plants all used a three step treatment of wastewater consisting of preliminary clarification, an activated sludge step and final clarification. The plants had a capacity of 15,000- 350,000 P.E. Mix of domestic and industrial or domestic and agricultural sewage. E2, E1 and EE2 were detected above the LOD in 14, 15 and 14 of 16 samples, respectively.	(*138*)

Table 5.1, continued. Concentrations of natural and synthetic estrogens in sewage effluent

Country	Compound	Conc. (ng/l)[1]	LOD (ng/l)	Method	Comments	Ref.
Germany	17β-estradiol estrone estriol ethinylestradiol	< LOD – 21 < LOD < LOD < LOD - 62	1 1 1 1	GC-MS	20 STPs. E2 detected above 10 ng/l in 2 and EE2 in 15 of the 20 samples.	(*143*)
Germany	17β-estradiol estrone ethinylestradiol	< LOD – 3 (< LOD) < LOD – 70 (9) < LOD – 15 (1)	1 1 1	GC-MS/MS	16 municipal STPs treating mainly domestic sewage. All STPs used at least three treatment steps: preliminary and final clarification and an aerator tank. E2, E1 and EE2 was detected in 8, 14 and 9 of 16 samples, respectively.	(*144*)
Sweden	17β-estradiol estrone ethinylestradiol	1.1 5.8 4.5	0.5 0.5 2	GC-MS	STP processing primarily domestic sewage from 3500 people. It had chemical and biological treatment steps – no anaerobic denitrification step.	(*3*)
Netherlands	17β-estradiol estrone ethinylestradiol	< 0.8 < 0.3 – 11 < 0.3 – 2.6	0.8 – 1.5 0.3 – 0.6 0.3 – 0.6	GC-MS/MS	Municipal effluent from five STPs (n=10)	(*6*)
Netherlands	17β-estradiol estrone ethinylestradiol	< LOD – 12 < LOD – 47 < LOD	0.1 – 1.8 0.1 – 1.8 0.1 – 1.8	GC-MS/MS	Three STPs sampled twice. All were based on activated sludge systems.	(*146*)
Netherlands	17β-estradiol estrone ethinylestradiol 17β-estradiol estrone ethinylestradiol	< 0.6 - 12 (0.9) < 0.4 - 47 (4.5) < 0.2 – 7.5 (< LOD) < 0.4 - 1.8 < 0.1 - 11 < 0.2 - 2.6	0.5-2.4 0.3-1 0.3-1.8	GC-MS/MS	Measured in effluent from three STPs receiving primarily domestic sewage (sampled twice). Total capacity of 300,000 – 560,000 P.E. Measured in two industrial STPs with a capacity of 100,000 P.E. All STPs had activated sludge systems	(*140*)
Italy	17β-estradiol estrone estriol ethinylestradiol	0.35 – 3.3 (1.0) 2.5 – 82.1 (9.3) 0.43 – 18 < LOD – 1.7 (0.45)	0.2 0.08 0.2 0.3	LC-ESI-MS/MS	From six Roman STPs (n=5 for each). The STPs received sewage from 40,000 – 1,200,000 inhabitants	(*145*)
Italy	17β-estradiol estrone estriol ethinylestradiol	< LOD – 7 < LOD – 54 < LOD – 28 < LOD – 2.2	0.1 – 1.8 0.1 – 1.8 o.1 – 1.8 0.1 – 1.8	GC-MS/MS	Five roman STPs (n= 2-3 for each). All based on activated sludge systems.	(*146*)

Table 5.1, continued. Concentrations of natural and synthetic estrogens in sewage effluent

Country	Compound	Conc. (ng/l)[1]	LOD (ng/l)	Method	Comments	Ref.
Israel	Sum of 17β-estradiol and estrone	6.5 – 50.1	1.91	RIA	Tel Aviv STP (n =3)	(*140*)
USA	17β-estradiol ethinylestradiol	0.48 – 3.66 < LOD – 0.76	0.107 0.053	RIA	Effluent from four municipal STPs E2 detected at all sites and EE2 at 3 of 4.	(*147*)
USA	17β-estradiol ethinylestradiol	< LOD – 4.05 < LOD – 2.42	0.1 0.1	ELISA	Effluent from four STPs (each sampled 4 times over a period of up to two years) with varying degrees of treatment up to very advanced.	(*148*)
Canada	17β-estradiol estrone estriol	< LOD 6 - 8 < LOD - 33	5 5 10	GC-MS	From four STPs (n=2 for each) High detection limits	(*149*)
Canada	17β-estradiol estrone estriol	< LOD – 64 (6) < LOD – 48 (3) < LOD – 42 (9)	1 1 1	GC-MS/MS	Ten municipal STPs treating mainly domestic sewage. All STPs used at least three treatment steps: preliminary and final clarification and an aerator tank. E2, E1 and EE2 was detected in 8, 8 and 9 of 10 samples, respectively.	(*144*)

E1: estrone, E2: 17ß-estradiol, EE2: ethinylestradiol
LOD: limit of detection.
[1] The figure in parentheses is the median concentration
P.E.: population equivalents
GC-MS: Gas Chromatography- Mass Spectrometry
LC.MS: Liquid Chromatography ElectroSpray - Mass Spectrometry
LC-ESI-MS/MS: Liquid Chromatography ElectroSpray Ion tandem Mass Spectrometry
HRGC-(NCI)-MS: High Resolution Gas Chromatography with Negative Chemical Ionisation Mass Spectrometric detection
RIA: radioimmuno assay
ELISA: Enzyme Linked ImmunoSorbent Assay
In some cases there are discrepancies between the LOD of the analysis and the actual lowest assessed concentration.
This probably exist because some runs allow a lower LOD than the most commonly used LOD.

Table 5.2. Concentrations of natural and synthetic estrogens in surface water.

Country	Compound	Conc. (ng/l)	LOD (ng/l)	Method	Comments	Ref.
Denmark	17β-estradiol estrone ethinylestradiol	< LOD – 0.8 0.2 – 3.0 < LOD – 1.5	0.4 0.4 0.5	GC-MS/MS	Concentrations in two lakes and three streams which receive no or little sewage effluent. Little non-treated effluent might come from sparse settlements.	(*2*)
England	17β-estradiol estrone estriol ethinylestradiol	<LOD – 7.1 0.2 – 17 < LOD – 3.1 < LOD	- - - -	GC-MS	Six sites on the river Thames sampled once. Low levels of estrogens were also found at a relatively unpolluted stretch of the Thames.	(*139*)
England	17β-estradiol estrone ethinylestradiol	< o.4 – 8.76 < o.4 – 12.22 < 0.5 – 4.6	0.4 - 1 0.4 - 1 0.5 - 1	GC-MS/MS	River Nene and Lea sampled for 28 and 14 consecutive days, respectively	(*35*)
Netherlands	17β-estradiol estrone ethinylestradiol	< 0.3 – 5.5 (< LOD) < 0.1- 3.4 (0.3) < 0.1- 4.3 (< LOD)	0.3 – 0.6 0.2 – 0.3 0.1 – 0.2	GC-MS/MS	Water samples from 11 coastal /estuarine and freshwater locations (n=3 for each). Highest concentrations detected at the freshwater locations River Rhine and Meuse. E2, E1 and EE2 detected in 4,7 and 3 of the sites, respectively.	(*145*)
Netherlands	17β-estradiol estrone ethinylestradiol	< 0.8 – 1.0 < 0.3 – 7.2 < 0.3 – 0.4	0.8 – 1.5 0.3 – 0.6 0.3 – 0.6	GC-MS/MS	Surface water from numerous streams, rivers, lakes, canals and marine areas (n = 97). E1 detected in 40-50 % of all samples. E2 and EE2 only detected above detection limit at each one site.	(*6*)
Germany	17β-estradiol estrone ethinylestradiol	< LOD < LOD – 1.6 < LOD	0.5 0.5 0.5	GC-MS/MS	15 German rivers and streams Only E1 detected (in 3 of 15 samples)	(*144*)
Germany	17β-estradiol estrone ethinylestradiol	0.15 – 3.6 (0.30) 0.10 – 4.1 (0.40) 0.10 – 5.1 (0.40)	0.15 0.1 0.1	HRGC-(NCI)-MS	31 water samples were analysed from the River Donau, and the Creeks Nau and Blau approximately 1 km downstream of STP effluent sites and from River Iller and three creeks. E2 , E1 and EE2 were detected above the LOD in 14, 29 and 15 of the 31 samples.	(*138*)
Germany	17β-estradiol estrone estriol ethinylestradiol	< LOD < LOD < LOD < LOD - 4	1 1 1 1	GC-MS	Five rivers. EE2 detected in 4 of 5 rivers.	(*143*)
Italy	17β-estradiol estrone estriol ethinylestradiol	0.11 1.5 0.33 0.04	0.02 0.008 00.2 0.03	LC-ESI-MS/MS	Measured in the Tiber River downstream of small towns whose sewages are treated by perculating filter STPs or directly into the river. No estrogens were detected in the river downstream of the city of Rome despite outlets of sewage from a number of STPs.	(*146*)

Table 5.2, continued. Concentrations of natural and synthetic estrogens in surface water.

Country	Compound	Conc. (ng/l)	LOD (ng/l)	Method	Comments	Ref.
USA	17β-estradiol ethinylestradiol	< LOD – 2.67 < LOD - 0.52	0.1 07 0.053	RIA	Water samples from 8 locations on Detroit river and 5 on Lake Mead, Michigan E2 detected in 10 and EE2 in 4 of 13 locations	(*147*)
USA	17β-estradiol ethinylestradiol	0.08 – 0.80 < LOD	0.05 0.05	ELISA	Sacramento Delta and Colorado river	(*148*)

E1: estrone, E2: 17ß-estradiol, EE2: ethinylestradiol
LOD: limit of detection.
[1] The figure in parentheses is the median concentration
P.E.: population equivalents
GC-MS: Gas Chromatography- Mass Spectrometry
LC.MS: Liquid Chromatography ElectroSpray - Mass Spectrometry
LC-ESI-MS/MS: Liquid Chromatography ElectroSpray Ion tandem Mass Spectrometry
HRGC-(NCI)-MS: High Resolution Gas Chromatography with Negative Chemical Ionisation Mass Spectrometric detection
RIA: radioimmuno assay
ELISA: Enzyme Linked ImmunoSorbent Assay
In some cases there are discrepancies between the LOD of the analysis and the actual lowest assessed concentration.
This probably exist because some runs allow a lower LOD than the most commonly used LOD.

5.1.2 Occurrence in surface water

The concentrations of estrogens which have been measured in surface water also show a large variability and concentrations of < 0.05 – 15.5 ng estradiol/l, < 0.1 – 17 ng estrone/l and < 0.053 – 30.8 ng/ ethinylestradiol/l have been detected (Table 5.2 and Chapter 4). Very few results exist again for estriol which has been detected at concentrations of < 0.1 – 3.4 ng/l. Though single very high concentrations of the estrogens have been detected they are generally found at concentrations of less than 5 ng/l for estradiol and estrone and less than 1 ng for ethinylestradiol. As for sewage effluent estrone is the most and ethinylestradiol the least frequently detected of the estrogens.

More studies are needed to assess the estrogen concentrations of surface waters since the use of the dilution factor in the receiving stream does not always give the right estimate of the actual surface water concentration when trying to estimate from sewage effluent concentrations. Kuch et al. 2001 found a dilution factor of only 2-6 of estrogens in the Danube River downstream of effluent discharge based on chemical analysis of the compounds though the effluent was diluted 100 times. This indicated that the river already contained a steroid load.

In Denmark estrogen levels have only been measured in surface water in a few streams and lakes which have served as reference locations and which did not receive or received sewage effluent in very low amounts (*2*). In these waters low levels of estrone could be detected in all samples in the range of 0.2 – 3.0 ng/l. Estradiol was detected less frequently at 4 of 5 sites at concentrations of 0.2 – 0.8 ng/l and a single detection of ethinylestradiol (1.5 ng/l) has also been made.
Estrone has been detected in upstream locations and in presumptive clean location in other studies (*139*). This indicates the need for also considering other sources of estrogens to the environment besides sewage effluent.

5.1.3 Fate and behaviour in the environment

When effluent is discharged to surface waters the aqueous concentration of estrogens will be reduced due to dilution, degradation and sorption of the compounds (*150*). Not much is yet known, however, about the fate, behaviour and persistence of steroidal estrogens following their discharge to the environment. Recent studies have begun to answer some of these questions (*35;36;150*).

5.1.3.1 Degradation in river water

Microorganisms in the environment are capable of degrading the estrogens. Using river water from urban/industrial and rural environments in England, the speed of biodegradation of estradiol and ethinylestradiol has been estimated. Estradiol was rapidly transformed to estrone under aerobic conditions in river water with a half-life of 0.2 – 8.7 days (at 20 °C) (*35;36*). Estrone was further degraded to non-estrogenic products at a similar halflife of 0.1 – 10.9 days. Mean half-lives of estradiol have been calculated to 2.8 days while mean half-life for estrone (calculated from 25 separately collected water samples) was 3.0 days (*35*).

The biodegradation rate of estradiol was temperature dependent and the fastest degradation was obtained with water samples collected in the summer

(half-lives of 4 – 5 hours). Incubation of river water at temperatures of 10 and 20 °C also demonstrated that half-life of estradiol was approximately twice as long at the lower temperature (*36*). The bacterial fauna will also influence the degradation rate.
The rate of removal seems to be independent of the estrogen concentration since the same rate was found for 100 ng/l to 100 µg/l of estradiol. Estadiol at 20 ng/l was also rapidly degraded indicating that low concentration of the hormone also will be metabolised even though high background levels of other organic carbon sources are present. Some studies have also indicated that degradation of estradiol is even faster at lower concentrations (*36;144*).

Ethinylestradiol is less biodegradable in aerobic river water and was shown to have a half-life of approximately 10 times that of estradiol under the same incubation conditions. In a comparison study, the half-life of estradiol and ethinylestradiol was assessed to 1.2 and 17 days, respectively (*35;36*).

All steroids have a low vapour pressure and are unlikely to volatilise from aquatic environments (*37*). Degradation by photolysis can, however, take place at slow rates and photolysis has been demonstrated to degrade estradiol and ethinylestradiol with a half-life of approximately 120 hours for both. Assuming 12 hours of bright sunlight this would equal 10 days.
Photolysis of estradiol is slow compared to the microbial degradation and considered unimportant for removal of this steroid in English rivers which are characterised by short transit time of the water (*36*). For ethinylestradiol this degradation pathway might play a larger role considered the slower biodegradation of this synthetic steroid.

5.1.3.2 Sorption to and degradation of estrogens in the sediment

When estrogens are released with effluent to the river water, a partitioning between the water phase and the sediment will take place (*150*). Sorption to suspended particles is also a removal route (*35;36;150*).
The log octanol-water partitioning coefficient (K_{ow} values) of estradiol, estrone and ethinylestradiol have been reported in the range of 3-4 and a little lower for estriol (<3) (*37;150*). A slightly higher K_{ow} value of the synthetic steroid has been detected (*150*) and these values therefore indicate medium sorption potential to organic matter in the following decreasing rank order: ethinylestradiol > estradiol = estrone > estriol (*37*).

Studies using natural sediments from three English rivers have also demonstrated the sorption of both estradiol and ethinylestradiol to both suspended and bed sediments with a slightly higher partition coefficient for ethinylestradiol (*35*). The amount and rate of sorption to the suspended material depend on its total organic contents and it is generally agreed that smaller particle size and higher organic content result in an increased sorption. The suspended sediment has been demonstrated to have a higher sorption efficiency than bed sediments (*150*). However, in English rivers less than 1 % of the present steroid are predicted to be removed from the water phase by suspended sediments when considering their concentration. Despite the lower sorption potential of the bed sediment, this might still be an important sink considering the available quantity (*35*).

The persistence of steroids in the bed sediment phase will depend on the oxygenation conditions. River bed sediments from heavily polluted rivers

might often be partly or completely anaerobic. Under anaerobic conditions estradiol has been shown to be rapidly converted to estrone. Using bed sediments from two English rivers, estradiol was degraded to estrone with a half-life of 8-16 hours. Estrone, on the other hand, seemed very persistent and no reduction was seen in the bed sediment concentration of this hormone over a period of 48 hours (*35;36*). If this results in a build-up of estrone in the sediment needs further investigation.

The conversion of estradiol in aerobic sediments was faster compared to the degradation taking place under anaerobic conditions with an approximately three times shorter half-life (*36*).

The degradation of ethinylestradiol under anaerobic conditions have been shown to be poor and this, together with the slightly higher sorption coefficient compared to that of estradiol, has led to speculations of whether ethinylestradiol might accumulate in bed sediments (*36*).

Sediments from an English sewage effluent receiving river have had concentrations that were 700-1700 fold higher than that of the overlying river water. This supports the suggestion that sediments are a major sink but also a source of estrogenic pollution (*151*).

Measurements of estrogens in the sediment of the English river Nene gave bed sediment concentration of estrone between 34 and 386 ng/kg while neither estradiol and ethinylestradiol were detected above the detection limit (100 ng/kg). The concentrations of estrone not only differed between sites but also at the same site on different days. Further studies on exact quantified sediment concentrations of estrogens are sparse.

5.2 Alkylphenols

5.2.1 Concentrations in sewage effluent

Concentrations of nonylphenol and octylphenol, the two most frequently encountered alkylphenols in sewage effluent, are in comparison with the natural and synthetic estrogens which are detected in the ng-range, in general detected at higher concentrations which sometime reaches µg/l levels (Table 5.3). Concentrations between 0.025 and 330 µg/l have been reported for nonylphenol and between 0.0022 and 73 µg/l for octylphenol although both, in general, mostly are found at concentrations below 10 µg/l. Levels below 4 µg/l have been detected in samples of effluent from Danish STPs whereas octylphenol seldom is detected at all (*2*).

Table 5.3. Concentrations of nonylphenol and octylphenol in sewage effluent

Country	Compound	Conc. (µg/l)[1]	Comments	Ref.
Denmark	NP OP	< 0.1 – 0.29 < 0.1	Eight sewage effluents. N = 3 for each. High-technology sewage treatment	(*2*)
Denmark	NP OP	< 0.o5 – 3.6 < o.1	Seven sewage effluents. Low technology sewage treatment	(*2*)
England	NP OP	1.2 – 2.7 0.030 – 0.190	One sewage effluent – primary domestic with 14 % industrial effluent	(*113*)
England	NP OP	< 0.2 – 330 < 1	Five STPS	(*152*)
England	NP	63	One sewage effluent. 6 – 10 % trade effluent from wool scouring and other textile industries Average conc.	(*34*)
Germany	NP OP	0.320 – 1.57 0.821 – 0.357	One sewage effluent	(*28*)
Germany	NP OP	0.025 – 0.770 0.0022 - 73	n = 16 (detected in 15 of 16 samples)	(*138*)
Sweden	NP	0.490	One domestic sewage effluent	(*3*)
Spain	NP	6 - 289	Effluent from four STPs	(*14*)
USA	NP	< 1 – 33 (<1)	20 STPs. 15 % of effluent was above LOD	(*153*)
USA	NP OP	16 0.15	One sewage effluent	(*154*)

NP: nonylphenol; OP: octylphenol.
[1] The figure in parenthesis is the median concentration

5.2.2 Concentrations in surface water

High levels of nonylphenols have been detected in some rivers in England, Switzerland and Spain in which concentrations between 45 and 644 µg/l have been reported (*14;152;155*). Most other reports on surface water concentrations of alkylphenols in European waters have not exceeded 1 µg/l (Table 5.4.). Octylphenol is typically
detected at lower concentrations in surface water with a maximum reported concentration of 13 µg/l in English river water (*152*).

Table 5.4. Concentrations of nonylphenol and octylphenol in surface water

Country	Compound	Conc. (µg/l)	Comments	Ref.
Denmark	NP OP	< 0.1 – 0.29 < 0.1	Two lakes and three streams without or with low sewage effluent outlets	(*2*)
Denmark	NP	< 0.05 – 0.100	Conc. in large streams in DK	(*156*)
England and Wales	NP OP NP OP	< 0.2 – 180 < 1 < 0.03 – 5.2 < 0.01 - 13	Six rivers. Highest conc. found in a highly industrialised river with inputs of surfactants form textile industry. Four estuaries OP detected only at one site	(*152*)
Germany	NP OP	0.001 – 0.221 0.0004 – 0.0036	Water samples from Elbe river and its tributaries	(*157*)
Germany	NP OP	< 0.010 – 0.485 <0.010 – 0.189	23 samples from five streams and rivers	(*158*)
Germany	NP OP	0.0067 – 0.134	N = 31 (detected in all samples)	(*138*)
Netherlands	NP OP	< 0.11 – 4.1 < 0.05 – 6.3	N = 97; - generally below the LOD for most surface water samples	(*6*)
Switzerland	NP	0.3 - 45	One river. Only one result was above 10 µg/l but 84 % were higher than 1	(*155*)
Spain	NP	0.15 - 644	2 rivers (downstream sites). Also detected at 18 – 51 µg/l upstream.	(*14*)
Canada	NP OP	< 0.01 – 0.92 < 0.005 – 0.084	N = 35. Both compounds were detected in 24 % of the water samples (especially in heavily industrialised and urbanised areas	(*159*)
USA	NP	< 0.11 – 0.64	30 rivers. Detected in 30 % of the samples.	(*160*)

5.2.3 Fate and behaviour in the environment

5.2.3.1 Degradation in river water

The alkylphenols, NP and OP are generally considered to be readily degradable by aerobic biotransformation in river water (*161-163*). Staples et al. 1999,2001 have reported biodegradation half-lives of 7 – 28 days (*162;163*). A lag time of approximately 2-3 days has been observed (*162;164*). A range of half-lives of 7 – 50 days was found when studying the biodegradation rate of OP in water from three English rivers (*161*). The fastest degradation was observed at urban/industrialised stretches of the rivers (half-lives of 8-13 days). As has been demonstrated for the estrogens, little difference in degradation rates was found for OP over a range of concentrations (20 – 100 µg/l) and OP is therefore not more persistent at low concentrations. Higher temperatures result in higher degradation (*163;165*).

5.2.3.2 Sorption to and degradation of alkylphenols in the sediment

Compared to estrogens, alkylphenols have a stronger affinity for bed sediments and therefore a higher tendency to accumulate in the sediment. The log K_{ow} has been reported to be 4.48 for nonylphenol and 4.12 for octylphenol which means that nonylphenol is most strongly absorbed to sediment and suspended particles (*166*). For octylphenol it has been demonstrated that the higher organic content and the greater proportion of clay and silt, the larger proportion of the alkylphenol will be sorbed to the

sediment. This is also in agreement with what has been demonstrated for estrogens.
In rivers with large amounts of organic aggregates, as has been seen at industrial reaches of English rivers, suspended sediments seem to play a large role in the fate of the octylphenol distribution. Here the suspended sediments absorbed 5-35 times more octylphenol than the bed sediments on a carbon-to-carbon basis and they had the potential to absorb 30-40 % of the octylphenol. In areas with low river velocity or large sedimentation rate as in estuaries the amount bound to the suspended particles will add to the concentration of the bed sediments.

The degradation in the sediment of alkylphenols has been reported to be very slow to practically non-existing in anaerobic environments (*117*). The half-life of nonylphenol in marine sediments in a Canadian study was estimated to be greater than 60 years. Long persistence has been shown in experiments where the amount of nonylphenol remaining after 440 days of addition was similar to the amount present in the sediment after 2 days (*38*).

In agreement with the high affinity to and the low degradability in sediment, high sediment concentrations for nonylphenol and octylphenol have been reported from numerous countries. In Swiss rivers, sediment concentrations of nonylphenol between 190 – 13,100 µg/kg dry weight have been detected (*155*). Sediment in a Korean bay which received industrial and municipal wastewater from two cities contained 113 – 3890 µg NP/kg dry weight and 3.97 – 179 µg OP/kg (*167*) and concentrations of <2.9 – 2960 µg NP/kg have been reported for sediment in US rivers. In UK estuarine sediments from highly industrialised areas concentrations of 1600 – 9050 µg nonylphenol and 30 – 340 µg OP/ kg dry weight were found (*168*) while sediment nonylphenol concentrations of 10-259 µg/kg dry weight and octylphenol concentrations of < 0.5 – 8 µg/kg have been reported from German rivers (*158*).

Bottom living fish which burrow in or feed on sediment therefore might be at higher risk towards exposure to alkylphenols than pelagic species.

5.3 Bisphenol a

5.3.1 Concentrations in sewage effluent

Existing reports on the level of bisphenol A in sewage effluent are not as numerous as seen for estrogens and alkylphenols. Levels of less than 1 ng to 6,2 µg/l have been reported but levels below 1 µg/l are most frequently encountered (Table 5.5). In Aarhus County,
Denmark levels of 1 ng/l to 4 µg/l in sewage from high technology sewage treatment works and up to 6,2 µg/l in sewage from plants with lower technology treatment steps have been found (*2*). This is within the range reported from other countries.

Table 5.5. Concentrations of bisphenol A in sewage effluent

Country	Conc. (µg/l)[1]	Comments	Ref.
Denmark	< o.1 – 4.0	Eight sewage effluents. N = 3 for each.	(*2*)
Denmark	< o.1 – 6.2	Seven sewage effluents. N = 3 for each. Low technology sewage treatment	(*2*)
Germany	0.018 – 0.702	39 sewage effluent	(*169*)
Germany	0.0048 – 0.047	N = 16. Detected in 15 of 16 samples.	(*138*)
Germany	0.16 – 0.36	One sewage effluent	(*28*)
Netherlands	< 0.043 – 4.09	Municipal effluent; n = 10	(*6*)
Sweden	0.490	One domestic sewage effluent	(*3*)
Canada	0.010 – 1.08 (0.136)	N = 34, municipal sewage effluent	(*170*)
USA	0.02 – 0.055	Two STPs (n=3 for each). Found in 3 of 3 samples	(*154*)

[1] The figure in parenthesis is the median concentration

5.3.2 Concentrations in surface water

Concentrations of bisphenol A in surface water are generally lower than in sewage effluent and have generally been detected in the range of < 0.001 – 1 µg/l (Table 5.6.). Downstream of sewage
from manufacturers levels up to 8 µg/l have been found. The levels seen in Danish streams were within the typical range reported for other European countries.

Table 5.6. Concentrations of bisphenol A in surface water

Country	Conc. (µg/l)	Comments	Ref.
Denmark	< o.001 – 0.44	Two lakes and three streams without or with low sewage effluent outlets	(*2*)
Germany	0.0005 – 0.41	116 samples from rivers, lakes and channels.	(*169*)
Germany	0.0005 – 0.014	n = 31. Found in all samples	(*138*)
Germany	< 0.050 – 0.272 (0.072)	23 samples from five streams and rivers	(*158*)
Germany	0.009 – 0.776	Water samples from Elbe river and its tributaries	(*157*)
Netherlands	0.0088 – 1	N = 97; found in almost all surface waters (fresh- and marine waters) throughout the Netherlands – except in larger bodies of water	(*6*)
Japan	0.01 – 0.268	Detected in 41 of 148 samples	(*171*)
USA	< 1 - 8	Downstream of manufacturers. Found at one of five sites.	(*172*)

5.3.3 Fate and behaviour in the environment

5.3.3.1 Degradation in river water

Bisphenol A is believed to be readily biodegradable in surface waters (reviewed in (*173*)),(*174*). A study has shown that bisphenol A in water samples was removed within 3-5 days with a half-life of 2.5 – 4 days (reviewed in (*173*)). Another study using surface water from both Europe and USA, from freshwater as well as estuarine environments, from light as well as heavily industrialised rivers, has shown a rapid degradation of bisphenol A with half-lives of 0.5 – 2.6 days. The biodegradation was

observed after a lag phase of 2 to 4 days. Half-lives of 3-6 days were detected in subsequent studies conducted with concentration of 0.05 and 0.5 µg/l bisphenol A. Disagreements exist as to whether microorganisms have to acclimate to the degradation of bisphenol A (*173;174*).

The biodegradation of bisphenol A results mostly in the production of the metabolites, 4-hydroxyacetephenone and 4-hydroxybenzoic acid which are rapidly converted to CO_2. Two minor metabolites, 2,2-bis(4-hydroxyphenyl)-1-propanol and 2,3-bis(4-hydroxyphenyl)-1,2-propanediol are also formed (*173*). 4-hydroxybenzoic acid is known not to have any estrogenic activity (*175*) while the other products, as far as is known, have not been tested.

Photodegradation of bisphenol A also seems to take place to some extent.

5.3.3.2 Sorption to and degradation of bisphenol A in the sediment

A log K_{ow} value of around 3.4 has been reported for bisphenol A (in (*173*)) which is within the same range as reported for the natural estrogens. This would also signify moderate sorption to the sediment. A study of anaerobic biotransformation of bisphenol A has, however, showed no loss of bisphenol A when incubated within 162 days under conditions promoting either methanogenesis, sulfate-reduction, iron(III)-reduction, or nitrate-reduction (*39*). Further, tetrabromobisphenol A, a widely used flame retardant, was shown to be completely dehalogenated to bisphenol A with no further degradation of bisphenol A under both methanogenic and sulfate-reducing conditions. This has indicated high potentials for accumulation of bisphenol A in anoxic sediments.

River sediment concentrations for bisphenol A have been reported from three German studies at < 0.5 – 15 µg/kg dry weight (DW)(*158*), 66-343 µg/kg DW (*157*) and 10 – 190 µg/kg fresh weight (FW)(*169*), respectively. A Dutch survey found sediment concentrations of < 1100 – 43000 µg/kg DW (*6*) while 2700 – 50300 µg/kg DW was found in a Korean bay which receives industrial and municipal wastewater from two cities (*167*). Measurements of bisphenol A levels in sediment have not been performed in Denmark.

6 Established dose-response relations between the estrogens/xenoestrogens and their feminising potential.

Following the observations of feminised male fish in sewage effluent receiving waters and the increasing evidence that estrogens and in some cases xenoestrogens are the compounds responsible for the observed endocrine disruption, a number of experiments have been performed to assess the lowest observable effect concentrations (LOECs) and no effect concentration (NOECs) for various feminising effects of estrogens and xenoestrogens on male fish. These studies have demonstrated that different species show different sensitivities towards the feminising potential of the various compounds. The LOECs and NOECs mentioned for vitellogenin induction in male and immature fish and for development of intersex or other reproductive effects in the following sections will in general be the ones found for the most sensitive species by water exposure studies. It is also important to note that LOECs and the NOECs often are dependent on the duration of exposure. Longer exposure periods have been seen to lower the limit for feminisation (*24;41*).

6.1 17β-estradiol

Experiments with juvenile rainbow trout has demonstrated that vitellogenin can be induced in this species after 14 days exposure to 4.7 – 7.9 ng/l estradiol (*40*). NOEC was in another study of the same author assessed to <5 ng/l (*41*).

Very low LOECs and NOECs have also been reported for induction of intersex or testis-ova in Japanese medaka (*Oryzias latipes*). In a group exposed to a nominal concentration of 10 ng/l estradiol for approximately 100 days from hatch and onwards 10 % intersex was found (*42*). No difference in sex ratio was found at this concentration. Exposure to 100 ng/l estradiol altered the sex ratio towards females and all males had developed the intersex condition. The NOEC for intersex induction concentration was assessed to 1 ng/l, but no concentrations between 1 and 10 ng/l were tested (*37*).

In another study, exposure of the same species for 28 days from hatch to 10 ng/l E2 produced all females (*43*). Complete sex reversal of genotypic male medaka to females have also been obtained when exposing to 1 µg E2/l from day 0-8 from fertilisation until hatch at 10 days post fertilisation (*176*). These results demonstrate that the timing of exposure in relation to the sex differentiation is important for the resultant effect on the expression of the sex.

Exposure of male fish to E2 has also resulted in a number of other reproductive effects. Miles-Richardson have found alterations in the morphology of seminiferous tubules of the testis in fathead minnow from

concentrations of 136 ng/l E2 after exposure for 14 days (*44*). Sertoli cells were hyperplastic and hypertrophied and degenerative changes such as loss of germ cells and presence of degenerated spermatozoa were observed. Electron microscopy revealed that the distended sertoli cells contained large phagolysosomes which contained various stages of degenerated spermatozoa and other cellular debris. This has also been seen after exposure of eelpout to E2 (*177*). An inhibition of spermatogenesis or inhibition of germ cell maturation was suggested in the study of medaka based on these observations plus the great number of earlier stages (spermatocytes) of spermatogenesis compared to controls (*44*). NOEC for these testicular effects was reported to 68 ng/l. Higher concentration of E2 (272 ng/l) also reduced the male secondary sex characteristics, fatpads and nuptial breeding tubercles.
In females changes in the follicular development such as a larger number of immature follicles has been observed after exposure to ≥ 27 ng/l. NOEC for this effect was 17 ng/l.

Some of the disruptions in the sexual development of fish caused by estrogens might be due to changes in the activity of aromatase, the enzyme responsible for the synthesis of estrogens from aromatisable androgens. Exposure of maturing fathead minnows to E2 caused on up-regulation of P450aromataseB mRNA expression in the testis and ovary in a dose-dependent manner after 14 days exposure (*45*). LOEC was 320 ng/l for males and 100 ng/l for females. In the male brain, P450aromataseB mRNA levels were further significantly elevated after 14 days exposure to 32 ng E2/l. GSI was reduced in males exposed to 100 ng E2/l.
In line with the indicated up-regulation of estradiol synthesis, a four times higher plasma estradiol level was found in male goldfish after exposure to 1000 ng/l E2 (*178*).

Overall, for 17ß-estradiol it can be concluded that although high exposure concentrations have been used to induce some alterations of the male reproductive system, a number of adverse effects have been seen at concentrations between 10 and 50 ng/l and even lower in regard to vitellogenin synthesis.

6.2 Estrone

Less studies have in general been performed with both estrone and estriol compared to the other natural estrogen, 17ß-estradiol.
The LOEC for estrone in regard to vitellogenin synthesis has been reported to be 3.2 ng/l for juvenile female rainbow trout (in (*37*)) after an exposure period of 14 days. Ten times as high a LOEC (31.8) was found for vitellogenin synthesis in male fathead minnow after 21 days of exposure (*47*) which compares well with a LOEC of between 25 and 50 ng/l for adult male rainbow trout exposed for the same period of time (*46*).

Induction of intersex in medaka has been obtained with a LOEC of estrone of 10 ng/l af 100 days exposure from hatch and onwards (*42*). No NOEC was assessed since 10 ng/l was the lowest tested concentration.
Testicular growth has been inhibited in male fathead minnow by 21 days of exposure to 318 ng/l (*47*).

Estrone is widely considered to be of similar or slightly lower *in vivo* estrogenic potency than estradiol as also seen from the above mentioned experiments. A 3-5 times lower potency of estrone has been suggested (*37*).

6.3 Estriol

The potency of estriol in regard to vitellogenin induction in male fish has not been determined by water exposure experiments. Estriol is in general considered to be the least estrogenic of the three natural estrogens. An i*n vitro* study has demonstrated estriol to be 30 times less potent than 17ß-estradiol (*42*).

In medaka exposed to estriol from hatch to 100 days after hatch a LOEC of 1 µg/l for induction of intersex was reported (*42*). One of 40 males had testes-ova at this concentration. At an exposure concentration of 10 µ/l all males had testis-ova.

6.4 Ethinylestradiol

A number of studies have examined the feminising potential of the synthetic estrogen, ethinylestradiol (EE2) on various endpoints. The NOEC for vitellogenin in fish has been tested in numerous species (*61;119;179-182*). Lowest concentration which have been found to induce vitellogenin in male fish is 0.1 ng/l (nominal concentration) after exposure of adult male rainbow trout for 10 days (*119*). Other studies with zebrafish and rainbow trout have reported vitellogenin induction at 1 – 5 ng/l EE2 in short-term exposure experiments (*50;61;66;179-181*). This agrees with the general finding that rainbow trout is the more sensitive of a number of test species with regard to vitellogenin induction (*181*).

Intersex in medaka has been observed after exposure to 0.1 ng EE2/l for 100 days from hatch (*42*). The assessment of this concentration as LOEC has been criticised (*37*) since intersex was only detected as a single oogonium within the testes of a single individual and no intersex individuals were observed at 10 ng/l. The concentration of 100 ng/l, however, clearly changed the sex ratio producing 91 % females and all males has testis-ova. Therefore a LOEC of intersex in medaka in this study has instead been proposed to be between 10-100 ng/L EE2 which is similar to the potency of estradiol reported in the same study. Another study with medaka obtained complete sex reversal with 100 ng EE2/l (*183*). In zebrafish, however, a change in sex ratio has been obtained at a much lower concentration (*48*). Exposing the fish to concentrations of 0.6 ng EE2/l from 20 to 60 day after hatch caused the sex ratio to change from an approximately 50:50 ratio to approximately 20:80 % (M:F).

In the sheepshead minnow (*Cyprinodon variegatus*) exposed for 59 days from a subadult stage to sexual maturity, LOEC and NOEC for induction of testis-ova or intersex were 20 and 2 ng EE2/l, respectively (*31*).
In a full life-cycle test on fathead minnow where newly fertilised eggs were exposed to EE2 for 305 days, a male:female ratio of 5:84 with intersex in 11 % of fish was seen at day 56 post hatch with an exposure concentration of 4 ng/l. A sex ratio of approximately 50:50 was seen in the control group (*184*). No testicular tissue was observed in any fish after 172 days post hatch and

male fish exposed to the same concentration failed to develop secondary sexual characteristics. NOEC for these effects was 1 ng/l.
Finally, though not a water exposure study, it should be mentioned that a single injection of 0.5 –2.5 ng EE2/egg has been shown to cause sex reversal in genetic male medaka (*185*). The sex reversed females had more atretic (degenerating) oocytes and fewer mature oocytes than unexposed females.

Other testicular effects such as fibrosis have been reported with a LOEC of 2 and NOEC of 0.2 ng EE2/l in the study with sheepshead minnow (*31*). An inhibition in the testicular growth seen as lowered GSI has been observed with 2 ng EE2/l in adult male rainbow trout (*50*)and with 10 ng EE2/l in zebrafish (*61;182*) after exposure for 21-24 days. Analysis of the distribution of the different developmental stages of germ cells in the rainbow trout and zebrafish testes revealed a higher proportion of the early stages compared to the stages in control fish (*50;182*). A similar altered pattern of germ cell development has been seen after exposure of newly fertilised eggs of fathead minnows to 10 ng EE2/l (in (*37*)). The fish were exposed at different time windows throughout the embryo development. Fewer spermatozoa in exposed male fish compared to control fish were also observed as was induction of the female duct, the ovarian cavity. The treatment, however, did not induce testis-ova.

The same concentration of 10 ng EE2 /l did in a 2 month exposure study with freshly hatched medaka result in aromatase activity in the testes of exposed male fish (*183*). The activity was normally only detectable in female medaka. As mentioned earlier aromatase is the enzyme which converts androgens to estradiol.

In conclusion, LOECs for vitellogenin and intersex induction by EE2 are very low observed at concentrations of 0.1 ng/l and a wide range of testicular effects have been seen at concentrations from 1-10 ng/l. This illustrates that ethinylestradiol is even more potent in regard to feminisation of male fish than 17ß-estradiol.

6.5 Alkylphenols

6.5.1 Nonylphenol

Xenoestrogens in general have a lower estrogenic potential compared to the natural and synthetic estrogens. Hemmer et al. found elevated vitellogenin levels in male sheepshead minnows (*Cyprinodon variegatus*) after 5 days exposure to 5.4 µg/l nonylphenol (*186*). No effect was found at a concentration of 0.64 µg/l. A LOEC in the same range (6.1 – 6.4 µg/l) has been found for juvenile rainbow trout following a 14 day exposure period (*40*). Long-term and intermittent exposure to nonylphenol can decrease the LOEC for vitellogenin synthesis, since exposure of adult male rainbow trout for ten days in every month for a total of 4 months caused vitellogenin induction by 1 µg NP/l (*51*). Exposure of juvenile rainbow trout for one year during embryonic, larval and juvenile life stages has also been demonstrated to increase vitellogenin expression in liver at 1.05 ng/l (*52*).
However, an example of an inverted dose-response curve for vitellogenin concentrations has also been reported in male fathead minnow exposed for 0.05 – 3.4 µg/l. Significantly higher vitellogenin levels were found among males exposed to the lowest nonylphenol concentration but not at higher concentrations (*187*). The effects of nonylphenol in this study were expected

to be caused by changes in the endogenous levels of estradiol since increased plasma levels of E2 were found in plasma at the lower nonylphenol concentrations. Only 4 % of the activity was estimated to be caused by the estrogen agonist activity of nonylphenol itself. This study therefore challenges the normal concept of dose-response studies.

Intersex has been induced in Japanese Medaka at nominal concentrations of 50 µg NP/l after exposure from hatch to 3 month of age. 50 % of the males had testes-ova at this concentration whereas 86 % showed the condition after exposure to 100 µg/l (*53*). The sex ratio was also significantly changed at this concentration to 1M:2F compared to 2M:1F in the control group.

A concentration of 30 µg NP/l has been capable of inhibiting testicular growth (lowered GSI) in mature male rainbow trout after an exposure period of 3 weeks (*50*). The inhibition was confirmed histologically by the predominance of less mature stages of sperm cells in exposed fish when compared to unexposed fish.
Degenerative effects on testes have also been seen after both high and low dose exposure to nonylphenol. Exposure of sexually mature male fathead minnow to 1.1 and 3.4 µg NP/l for 42 days caused necrotic aggregates of various stages of germ cells and the presence of phagocytic cells (*54*). Higher dose exposure (100 µg/l) of adult male medaka has been demonstrated to result in a six-fold greater extent of apoptosis (programmed cell death) in spermatocytes, sertoli cells and leydig cells (*188*) and similar observations of apoptosis and degenerated cells in interstitium and in cell types in the seminiferous tubules along with suppressed spermatogenesis have been observed after just 3 days exposure of swordfish (*Xiphophorus helleri*) to the same concentration (*189*). Longer exposure for 60 days resulted in reduced sword length, a secondary sexual characteristic in males used to attract females. Abnormal, female-like anal fins have also been induced in male medaka after exposure to 100 µg NP/l 200-230 days from the embryonic stage. Abnormal testes were also seen in this study by Tabata et al. 2001.

Importantly, transgenerational effects of nonylphenol have also been observed since exposure of both sexes of adult fathead minnows intermittently for four months prior to spawning for 1 and 10 µg/l resulted in a two-fold increase in the plasma level of estradiol in plasma of male offspring and a 13-fold increase in the testosterone level in plasma of female offspring (*51*).

6.5.2 Octylphenol

Octylphenol has generally been reported to have a higher estrogenic potential than nonylphenol when compared in the same test system (*50;190*).
Lowest LOEC which has been reported for octylphenol is, however, not different from the one reported for nonylphenol. This discrepancy might be due to fewer studies performed with octylphenol than nonylphenol. In a three week exposure study with rainbow trout vitellogenin was induced by 4.8 µg/l with a reported NOEC of 1.6 µ/l (*50*). The concentration of 4.8 µg OP/l reduced GSI.

Development of intersex in medaka and a shift in sex ratio towards females have been found with 2 - 50 µg/l OP when exposing from hatch to maturation (*49*). Concentrations greater than 41 µg/l have resulted in an inhibition of testicular growth in the same species, seen as an increase in the early

spermatogenetic stages (*191*). In rainbow trout a similar skewed germ cell distribution has been induced by 30 µg OP/l. Testicular fibrosis in adult medaka has been caused by 100 µg OP/l (*192*).

As described in section 10.3 alkylphenols are degradation products of alkylphenolpolyethoxylates – a degradation which takes place in the sewage treatment process. Other degradation products such as alkylphenolmono- and –diethoxylates and alkylphenolmono- and diethoxycarboxylates are also formed (*193*). Compared to alkylphenols, nonylphenolmonoethoxylate and nonylphenoldiethoxylate have in some studies been shown to have approximately equal potency to nonylphenol but lower potency than octylphenol (*50;194*) while other studies have found weaker estrogenic activity of these short chained ethoxylate- and carboxylate-derivatives compared to the alkylphenols (*42;54;195;196*). *In vitro* the ethoxylates and carboxylates have been demonstrated to be 10^4-10^5 less potent than 17ß-estradiol (*42;195*). *In vivo* 100 µg/l of either nonylphenolmono- or diethoxylate or nonylphenolmono- or diethoxycarboxylate could not induce intersex in medaka (*42*). In general, however, little knowledge exist on the estrogenic activity in fish of these compounds compared to nonylphenol and the role of these degradation products of alkylphenolpolyethoxylates in the total emission of estrogenic chemicals is not clear. They might not be disconsidered in the total picture of estrogenic chemicals in sewage effluent.

6.5.3 Bisphenol A

Bisphenol A is less potent as an estrogenic agonist compared to the alkylphenols. In rainbow trout the LOEC for vitellogenin synthesis in males has been found to be between 40 and 70 µg/l (*55*). Occurrence of intersex has, however, been reported in male medaka exposed to 10 µg/l for approximately 100 days from hatch (*42*). In fathead minnow 16 µg/l bisphenol A reduced the number of mature spermatozoa produced by sexually mature males after 164 days exposure (*56*).

In general higher concentrations seem to be needed to produce reproductive effects by bisphenol A. Inhibition of gonadal growth was observed in male medaka with a LOEC of 640 µg/l (*56*) and degenerative and necrotic effects on germ cells which have been seen with 100 µg/l NP exposure of swordfish were not seen with a concentration of 10 mg/l bisphenol A (*189*).

6.5.4 Phthalates

The health risk associated with the use of phthalates has recently received much attention in the Danish media. It has long been known that phthalates are testicular toxicants (*197;198*) but they do not appear exclusively to exert their action though a direct estrogenic mechanism in which they bind to the estrogen receptor. *In vitro* only 5 out of 35 of the commercially most used phthalates have been demonstrated to have a very weak estrogenic activity (*199*). The phthalate, butylbenzylphthlate (BBP), which has been demonstrated to have the highest estrogenic activity, was 1-milllion-fold less potent than 17ß-estradiol *in vitro* (*199*). This is also the only phthalate which has been demonstrated to have a very weak *in vivo* estrogenic activity in fish and this only at environmentally unrealistic doses (*41*). Some phthalates have also been demonstrated to have antiandrogenic activity (*200*). The most widely used phthalate, diethylhexylphthalate (DEHP) was in the *in vitro* study above not found to have any estrogenic activity (*199*). A single study has with

caution identified DEHP as a possible contributor to *in vitro* estrogenic activity in a water sample from an English estuary but also points the possibly that this might be due to contamination. DEHP is a common laboratory contaminant (*129*).

Since the present report concentrates on estrogens and estrogenic chemicals which exert their effects by mimicking estradiol and also considering the very weak estrogenic activity of phthlates, they are not included in this report.

7 Relation between environmental levels of estrogens/xenoestrogens and known effect concentrations.

When comparing the concentrations of natural and synthetic estrogens in effluent and surface water with the dose-response relationships for the various reproductive effects described in the previous chapter, it is apparent that some concentrations have been found in various effluents and also in some aquatic environments which are above the LOECs described for i.e. induction of vitellogenin synthesis in male fish, induction of intersex and for causing other testicular effects. Concentrations of < 0.1 – 88 ng/l and 0.05 – 15.5 ng/l have been reported for estradiol in sewage effluent and surface water respectively. LOECs for vitellogenin induction and intersex have in comparison been reported to 5 and 10 ng/l and a range of other testicular effects have been seen at 10 – 50 ng/l. When the estrogen concentration has only been reported in sewage effluent it is, however, difficult to estimate whether this causes a resultant surface water concentration above the reported LOEC, since dilution and degradation in relation to the flow of the river will affect the fate of the steroid and therefore the final water concentration. Still, some reported surface water concentrations of estradiol have been high enough to predict feminisation of male fish.

For estrone, concentrations in the range of <0.1 – 220 ng/l in sewage effluent and <0.1 – 17 ng/l in surface water have been detected and LOEC for induction of vitellogenin in male fish has been reported to approximately 30 ng/l (ten times lower for juvenile females). LOEC for induction of intersex in males has been reported to 10 ng/l which was the same as found for estradiol. Judged from these observations estrone levels in surface waters can be a candidate for causing feminisation of fish – also given the higher frequency of detection of this steroid compared to the other estrogens.

Whether or not the other natural estrogen, estriol, participates in some of the observed reproductive disturbances reported from numerous countries is as yet not possible to assess since analyses of effluent and surface water concentrations plus *in vivo* water exposure experiments on estriol are sparse. One study has found a LOEC of intersex induction in medaka of 1 µg/l which is far above the few existing reports on estriol concentrations in surface water (< 0.1 – 3.4 ng/l) and sewage effluent (< 0.1 – 42 ng/l). Data on this steroid must, however, be considered inadequate for a reliable judgement of its contribution to the feminisation of male fish. As mentioned earlier *in vitro* studies have found estriol to be 30 times less potent than estradiol (*42*).

Ethinylestradiol is generally detected less often than both estrone and estradiol and although it has been found in the range of < 0.1- 62 ng/l in effluent and 0.053 – 30.8 ng/l in surface water it is mostly detected below 5 ng/l (and often below 1 ng/l). Very low concentrations of the synthetic hormone are, however,

capable of causing feminisation of male fish. Both vitellogenin induction and intersex have been reported at 0.1 ng/l, and although some have questioned the LOEC of intersex of that particular study (*37*) another experiment has demonstrated changed sex ratios at concentrations down to 0.6 ngEE2/l. A number of other testicular effects such as reduced testicular growth and inhibition of spermatogenesis have also been found at concentrations below 10 ng/l. Since the detection limit for many chemical analysis for EE2 is between 0.1 and 1 ng/l this impedes the judgement as to whether the concentrations in the environment is a risk factor in relation to feminisation of male fish. In some European waters ethinylestradiol has, however, been detected in concentrations which could explain all or part of the observed feminisation (*3;46*).

Alkylphenols have in some hot-spot areas of England also been suspected as primary or contributory compounds to feminisation of male fish (*34*). Internationally, sewage effluent concentrations between 25 ng/l and 330 µg/l and surface water concentration between 5 ng/l to 180 µg/l have been reported for nonylphenol. A single river water concentration above 600 µg/l has been reported from Spain. Typically, concentrations in sewage effluent and surface water does, however, not exceed 10 and 1 µg/l, respectively. In the rivers with high concentrations of nonylphenol, these exceed well the LOEC for vitellogenin synthesis which is approximately 5 µg/l, and a long-term exposure study has moved the LOEC down to 1 µg/l (*51*). Exposure to between 30 and 100 µg/l has further resulted in inhibited testicular growth, changed sex ratio and degenerating testes. Degenerated testes have also been observed at low NP concentrations below 5 µg/l. In some rivers, nonylphenol will therefore be a likely endocrine disrupting compound.

Octylphenol, as mentioned earlier, is generally assumed to have a higher estrogenic potency than nonylphenol but it is not as widely used in industry as nonylphenol. This is also reflected in the concentrations of octylphenol found in both sewage effluent and surface water in which it is found in concentrations of between 22 ng/l and 73 µg/l and 0.4 ng/l – 13 µg/l. The high end concentrations are above the LOEC of vitellogenin synthesis of 5 µg/l (*50*) and above the concentration which have caused intersex and a shift in sex ratio in the medaka (2µg/l) (*49*), and octylphenols might have participated in feminising effects in rivers with high concentrations.

Few reports exist as mentioned on bisphenol A which together with alkylphenols are among the most potent of the presently known estrogenic chemicals. Highest concentrations reported in effluent and surface water is 6 and 1 µg/l, respectively with a single observation of 8 µg/l in a river site downstream of a manufacturer. Most effects of bisphenol A have been reported at concentrations above 40 µg/l, though intersex and a reduced number of spermatozoa have been found at exposure concentrations of 10 – 20 µg/l. Since only a single report exist of bisphenol A in concentrations near the lowest LOEC for any effects, this xenoestrogen is considered to play little or no part in the feminising effects observed at fish in different parts of the world.

When assessing the possible implications for the reproductive health of male fish of the estrogenic compounds in the aquatic environment it is, however, important not only to look at the individual levels of single estrogens or xenoestrogens. The different estrogens and xenoestrogens will act in a concerted manner with an additive activity (*40*). This means that

concentrations below the LOECs for the individual compounds can exert an estrogen activity when present in a mixture of other estrogens and xenoestrogens. This has been demonstrated for both the combination of estradiol and ethinylestradiol (*57*) and estradiol and nonylphenol (*40*) in regard to vitellogenin synthesis in rainbow trout.
The estrogenic potency of mixtures in sewage effluent and the surface water is therefore important in evaluating the risk for the aquatic fauna.

Another aspect which has to be taken into consideration when assessing the risk of sewage effluent outlet to the environment, is the consequences of intermittent release of high concentrations of endocrine disrupting compounds in pulses. Exposure of fathead minnow to shorter, repetitive high concentrations of estradiol resulted in concentrations of plasma vitellogenin which were higher than continuous exposure to the equivalent time-weighed average concentration.

In general, therefore both the capacity of single estrogens and estrogenic chemicals, the additivity of the compounds with the same estrogenic mechanism of action and the unproportionate effects of intermittent exposure to high concentrations of the compounds have to be taken into account when evaluating the compound(s) responsible for the total estrogenicity of sewage effluent and the compounds responsible for the already observed endocrine disruption of the male fish reproductive system.

It is also important to bear in mind that many of the controlled laboratory studies performed to assess LOECs and NOECs for the various estrogenic compounds often are short-term exposure experiments. As mentioned earlier long-term exposure experiments have been demonstrated to lower the concentration needed to result in testicular or other effects such as vitellogenin synthesis. Chronic exposure to low levels of estrogens might result in reduced LOECs for the different endocrine disrupting effects. Further, the effects of an exposure may depend on the timing of the exposure in relation to the sex differentiation and reproductive cycle of the fish. The most sensitive stage in regard to disruption of the reproductive system is generally thought to be the very early life stages in which the sex is determined and differentiated (*58*). The effects created at this stage is also often irreversible since it involved the formation of for instance female somatic structures. There might, however, also be periods in the reproductive cycle of adult, sexually mature fish in which they are more susceptible to estrogen exposure than others.

8 Consequences of feminisation on the reproductive success/fertility of male fish.

An important question which has been raised after the observations of reproductive disturbances in male fish in the environment is, whether these effects will have subsequent detrimental consequences for the fertility or reproductive capacity of the male fish and whether this will have effects on the population level. This question is difficult to answer based on the present observations.

New results have recently been published on the reproductive health found among British intersex roach captured downstream of STPs (*60*). An asynchrony of gamete maturation among sexes was observed. When examined in autumn, in the middle of the reproductive cycle of the roach, the spermatogenesis was delayed in intersex and male fish from the sewage effluent receiving rivers and growth of the testes was inhibited in the most severely intersexed fish. In sewage effluent affected females, a higher incidence of oocyte atresia (degeneration) was found, and in both intersex males and females changed sex hormone levels were observed. When examined in spring around the time of spermiation only approximately 50 % of the males from the two sewage effluent receiving rivers were able to release sperm compared to 100 % spermiation among males from control sites. Further, those intersex fish which did spermiate had a reduced milt volume and a reduced sperm density. The lack of ability to spermiate could in some males be explained by observations of abnormalities in the sperm duct which could prevent the release of gametes. Female roach at this sampling time had already begun to spawn and these observations of asynchrony in the gamete maturation therefore indicate reduced reproductive success among the roach population from these rivers.

In vitro fertilisation studies with gametes from roach taken from the effluent receiving rivers have supported this indication of reduced fertility (*57*). The viability of the gametes of wild roach was reduced for both male and female roach – but particularly for sperm and there was a negative correlation between the degree of intersex in an individual and the fertilisation success. In general the proportion of surviving embryos fell at each developmental stage but this pattern was magnified in embryos produced by severely intersexed fish.
The factors that seemed to be responsible for the reduced male fertility among intersex fish were reduced sperm density, a lower proportion of motile sperm and reduced duration of sperm motility.

The above mentioned studies are the only ones which yet have been performed to assess the effects of the observed feminisation on the reproductive capacity of wild male fish. A few studies with controlled exposure to estrogens and xenoestrogens have given some additional information as to whether these compounds have the potential to reduce the fertility.

A fertilisation success below 70 % was observed in male zebrafish at exposure concentrations of 5-25 ng/l ethinylestradiol (*61*). In addition, the number of non-exposed females which spawned successfully when paired with exposed males was below the expected breeding success of non-exposed pairs. Therefore both the fertilisation success and the sexual behaviour of the exposed male zebrafish seemed to be impaired. At exposure to 10 and 25 ng/l EE2, significant reductions in the GSI, signalling altered spermatogenesis, were seen. This indicates that for some species such observations of inhibited testes development are indicatives of reduced fertility.

A lower fertilisation rate (though not significant) has also been found for medaka when males exposed to OP (2-50 µg/l) or E2 (100 ng/l) were paired with unexposed females (*49*).
In male medaka exposed to a higher concentration of E2 (817 ng/l) for two weeks decreased or disappeared spermatogenesis was observed (*62;63*). Pairing the exposed males with unexposed females resulted in a decreased number of eggs laid by females and a decrease in the number of hatchlings. Bisphenol A caused a decrease in total egg number and hatchlings at a concentration of 2283 µg/l, while a decrease in the number of hatchlings was seen with 66 µg/l nonylphenol. In a similar study where male madaka was exposed to 20 – 280 µg/l octylphenol, unexposed females paired with these males produced approximately 50 % fewer eggs than the control group (*191*).
Exposing both male and female rainbow trout to 10 µg NP/l for 10 days in each of 4 consecutive months prior to spawning resulted in reduced hatching rate of the F1 offspring due to high mortality of eggs before the eyed stage (*51*).

A commonly observed feminisation in male fish which as described earlier has been observed in the field (and also in multiple controlled exposure experiments with estrogens and xenoestrogens) is the production of the female yolk protein, vitellogenin. The significance of this in relation to the reproductive capacity of the male fish is not known. Further, it is more difficult to interpret the importance of the vitellogenin production in males in relation to reproductive success than for instance a reduced capacity to fertilise eggs.
High levels of vitellogenin in male fish have been reported to cause acute renal failure due to excessive accumulation of vitellogenin in the kidney since the male fish in contrast to females don't have a target organ for the protein (*64;65*). Chronic exposure to lower concentrations of estrogenic chemicals might not cause the extensive liver and kidney pathologies as has been observed with high concentration exposures, but it has been proposed that a milder effect on the liver and kidney might result in a reduced ability to metabolise xenoestrogens or to resist diseases (*65*). Further, it has been suggested that vitellogenin production in males may decrease the part of metabolic consumption which is used for growth and spermatogenesis (*66*) and in females may result in altered calcium metabolism and therefore altered growth and bone formation in the embryos or larvae (*201*). A study by Gronen et al. 1999 has found a negative correlation between vitellogenin levels of octylphenol exposed male fish and percent fertilised eggs when these where paired with unexposed females (*191*).
Both EE2 and NP have also been shown to cause severe anaemia in common carp exposed over a 70 day period to 500 µg/kg or 1 to 15 µg/l, respectively – supporting the fact, that a toxic response of a xenoestrogen or estrogen indirectly might influence the capacity left for sound reproduction due to an impairment of the general health condition of the fish.

9 Effects of (xeno-)estrogens on the reproductive success/fertility of female fish.

Most studies conducted both in the field and in the laboratory have focused on the feminising potential of estrogens and xenoestrogens on the male reproductive system and the consequences for the male fertility since the most detrimental effects are suspected to be seen in the male gender due to its low endogenous levels of estrogens. During the last few years some attention has, however, also been given to the effects of the compounds on the female fertility which have demonstrated that relatively low concentration of both steroid estrogens and xenoestrogens also are capable of reducing the reproductive success of female fish.

In female roach from the English rivers, in which high frequencies of intersex have been found among males, atretic and vacuolated oocytes were found.
In female zebrafish a dose-related reduction in the number of spawning females were observed at three weeks exposure to 10 ng EE2/l with complete inhibition of spawning at levels of 25 ng/l (*61*). The non-spawning females had significantly smaller ovaries lacking mature oocytes, indicating that EE2 interfered with oocyte maturation.
Another study with zebrafish resulted in reduced egg production in females exposed to 10 and 25 ng/l EE2 when exposing for a period of 4 month from the embryo stage (*68*). In fathead minnow exposure to 10 ng/l EE2 has also caused a reduction in the number of oviposited eggs after a 4-week exposure period (*202*). In a partial life-cycle test of the marine species, sheepshead minnow, a LOEC for reduction in the egg production was observed by 20 ng EE2/l in one spawning trail and by 200 ng/l in a second (*31*). Hatching success was also reduced among the progeny of fish exposed to 200 ng/l. Both males and females were exposed in this study and it was therefore possible that the reduced reproductive success was a consequence of both atresia of oocytes in the ovaries and extensive fibrosis of the testes observed at this concentration.

Exposure studies on female with E2 have found reduced egg spawning in medaka at 27.2 ng/l (*63*) and an EC_{50} for inhibition of egg production in fathead minnow at 120 ng E2/l (*203*).

Nonylphenol exposure has also reduced the egg production of females. This was seen in medaka after exposure for two weeks to 6.6 µg NP/l. In rainbow trout exposure of female fish for 18 months during early ovarian development to 85.6 µg NP/l shut off the reproduction of the female totally, since vitellogenin, although produced in large quantities, was not taken up by the oocytes (*69*). The oocytes therefore did not develop further. A possible explanation for this might have been the concomitantly observed suppression of follicle stimulating hormone (FSH) synthesis in the pituitary and reduced FSH level in the plasma. This gonadotophin is thought to induce the recruitment of oocytes into maturation and stimulates the uptake of vitellogenin into the developing oocytes. Reduced FSH levels in plasma were

suppressed with lower concentration of NP (0.7 and 8.3 µg/l) but changes in the ovarian development was not observed at these concentrations.

A study with estradiol has also demonstrated that vitellogenin can autoregulate its own synthesis by the liver in females via down-regulation of the E2-production of the oocytes (*204*). Vitellogenin synthesis is as earlierdescribed induced during the normal female reproductive cycle by estradiol and synthesised in the outer follicular layer of the oocytes. Female rainbow trout injected with E2 had disturbed development of the ovary and had delayed sexual maturity compared to controls. Sustained high circulating levels of vitellogenin before the actual period of natural vitellogenesis, which can be induced by exogenous exposure to estrogens or xenoestrogens, might therefore prevent a normal development of the oocytes.

No studies have been published which have concentrated on the effect of sewage effluent exposure on the fertility of female fish.

10 Potential sources of estrogens and xenoestrogens

In light of the observed feminisation of fish and the increasing evidence that estrogens and/or xenoestrogens in sewage effluent are possible sources to the effects, the following chapters will focus on the sources of the estrogens and xenoestrogens. The fate of the compound in the sewage treatment plants and the effect of different treatment types on the release of estrogens will also be described.

10.1 Estrogens

Estrogens are female steroid sex hormones based on a cholesterol skeleton. Estrogens are produced naturally in vertebrates in the gonads and adrenal cortex of both sexes and the synthesis goes via progesterone and androstenedione or testosterone of which the latter two are male sex hormones (androgens). Both estrogens and androgens are present in both sexes but in males the androgens dominate while estrogens dominate in the females.

The follicle-stimulation hormone controls the excretion of sex hormones in the organism and the luteinizing hormone released from the pituitary gland. The production and excretion of sex hormones change throughout the life of humans. For both male and female the production of sex hormones is small until puberty is reached. After puberty the production of testosterone in men increases gradually until an age of around 40 years is reached, after which it starts to decrease (*205*). The testosterone is altered to estradiol in some parts of the body, e.g. the brain (*74*).

10.1.1 Men

Measurements of the total estradiol in the blood stream of three age groups of men showed the following average levels: 36-55 yr men: 0.0073 µg/l, 56-66 yr. men: 0.020 µg/l, and 66-80 yr. men: 0.026 µg/l (*206*). Estimates of estrogens excreted in male urine are shown in Table 10.1.

Table 10.1.Excretion of estrogens in urine by males (72)

	Excretion
Estrogen	[µg/24 h]
Estriol	1.5
Estrone	3.9
Estradiol	1.6

10.1.2 Women

For women the production of estrogens is more complicated. After sexual maturity the production of estrogens varies during the menstrual cycles and the pregnancy until the menopause is reached.

10.1.2.1 Menstruating women

During the menstrual cycle, plasma concentrations and excretion of estrogens increase from day one to reach a maximum a couple of days before ovulation (day 14). After this period, the excretion declines until a couple of days after ovulation. At that time, the production by corpus luteum causes an increase of the excretion again (luteal phase). If no fertilization of the egg occurs then the corpus luteum degenerates, the estrogen excretion decreases and the woman will menstruate. The excretion of estrogens in urine during the menstrual cycle as found by Brown (1955) is shown in Table 10.2.

Table 10.2. Excretions of estrogens in urine by menstruating women

	Menstrual phase			
Estrogen [µg/24 h]	Cycle start	Ovulation peak	Luteal phase	Average
Estriol	6 (0-15)	27 (13-54)	22 (8-72)	18
Estrone	5 (4-7)	20 (11-31)	14 (10-23)	13
Estradiol	2 (0-3)	9 (4-14)	7 (4-10)	8

(*207*). Average calculated in the present report.

Results from more recent studies of the excretion of estrogens have been collected by Johnson et al. (*72*), (Table 10.3.

Table 10.3. Average excretion of estrogens in urine by menstruating women (*72*)

	Excretion
Estrogen	[µg/24 h]
Estriol	4.8
Estrone	8.0
Estradiol	3.5

The values of Johnson et al. (*88*) are used in an estimation of the total emission of estrogens.

10.1.2.2 Pregnant women

During pregnancy, first the corpus luteum and later the placenta produces large amounts of estrogens and the levels in urine increase. The urine excretion rate of estrogens and progesterone increases steadily during pregnancy reaching maximum at delivery as shown in Table 10.4. **(74).**

Table 10.4. Excretions of estrogens in urine by pregnant women

Estrogen [µg/24 h]	Menstrual phase		
	8 weeks	16 weeks	24-40 weeks
Estriol	100	1000	25000
	0-20 weeks	22 weeks	40 weeks (delivery)
Estrone and estradiol	100-500	1000	2000

(*73*)

Johnson et al. (*72*) have reported average values of the excretion as given in Table 10.5.

Table 10.5. Average excretion of estrogens in urine by pregnant women (*72*)

	Excretion
Estrogen	[µg/24 h]
Estriol	6000
Estrone	600
Estradiol	259

These values are in agreement with the values reported by Frandsen et al. (*73*) and are used for estimation of total emission.

10.1.2.3 Post menopausal women

At the menopause, which usually occurs between the age of 45 and 55, in Denmark (on average 51), the menstrual cycles stop and the production of estrogens by the ovaries ceases. Post menopause production of estrogens is very low (*74*). It has been estimated as shown in Table 10.6.

Table 10.6. Average excretion of estrogens in urine by post menopausal women (*72*)

	Excretion
Estrogen	[µg/24 h]
Estriol	1.0
Estrone	4.0
Estradiol	2.3

10.1.3 Metabolism

Almost all estrogens are emitted as glucuronides, little as sulphates and less than 4 % in unconjugated form.

The estrogens are metabolised mainly in the liver but also elsewhere. Estradiol is both metabolised reversibly and irreversibly. In the reversible metabolism, estradiol is transformed to estrone and estrone sulphate. These circulate in the blood stream and act as estradiol reservoirs. In the irreversible metabolism, estradiol is transformed to cathecol estrogens or estriol. The metabolites from both reversible and irreversible metabolism are, to a large degree, finally conjugated with sulphate and glucuronides and excreted in the urine (Table 10.7.). A minor amount of the estrogens are excreted via faeces as un-conjungated metabolites (*70;71*).

Table 10.7. Distribution of estrogens excreted by non-pregnant women in urine.

	Distribution
Estriol (conjugated*)	20-30 %
Estrone (conjugated)	10-20 %
16α-hydroxyestrone (conjugated)	5-15 %
Estradiol (non metabolised)	5-15 %
16-epi-estradiol (conjugated)	6 %

2-methoxyestrone (conjugated)	5 %

*Conjugated forms are glucuronides and sulphates

However, the conjugation is reversible. In the organism, conjugated metabolites are to some degree hydrolysed in the intestines and reabsorbed (*70*). This may very well also take place outside the organism, e.g. in waste water, and unconjugated estriol, estrone etc. may thus be formed.

10.1.4 Hormone therapy

10.1.4.1 Estrogen replacement therapy

Estrogen replacement therapy may be given to women who have entered the menopause or who have had their uterus and ovaries removed.

It may be given in various forms, as outlined in Table 10.8.

Table 10.8. Administration of estrogen replacement drugs (*205*)

Administrative form	Estrogens
Oral (pills or tablets)	Conjugated estrogens, estradiol, esterified estrogens, estropipate, ethinyl estradiol.
Transdermal (patch, placed on the skin, that releases estrogen continuously):	Estradiol
Vaginal cream (cream inserted into the vagina that releases estrogen continuously):	Conjugated estrogens, estradiol, estropipate
Vaginal ring (ring inserted deeply into the vagina that releases estrogen continuously for 3 months):	Estradiol

10.1.4.2 Hormone replacement therapy

Hormone replacement therapy includes the administration of two hormones estradiol and progestin (acts like progesterone). It may either be given as combination drugs or progestin may be given supplemental to the estrogen replacement therapy drugs (Table 10.9.).

Table 10.9. Administration of hormone therapy drugs (*205*)

Administrative form	Combination
Pills or tablets	Conjugated estrogens/medroxyprogesterone acetate
Transdermal combination preparations (a patch placed on the skin that releases estrogen and progestin continuously):	Estradiol/norethindrone acetate
	Progestin supplemental
Pills or tablets	Medroxyprogesterone acetate, norethindrone, norethindrone acetate, micronized progesterone

Orally administered estradiol or estrone are mainly excreted in the urine. Recoveries of 50-80 % in the urine have been shown, and up to 18 % in faeces, after 4-6 days (*208*). The values of 65 % in urine and 15 % in faeces are used for estimation of excretion in this report. Estrone and estradiol constitute 1/3 of the estrogen amount in faeces (*209-211*).

10.1.4.3 Hormone contraceptives

Birth control pills (oral contraceptives) contain hormones and are taken daily to prevent pregnancy.

Combination birth control pills are the most commonly used. They contain ethinylestradiol and progestin (*205*). However, the so-called "mini-pills", which only contain progestin, are also used. Several types of emergency contraception pills exist and some contain the hormones: Progestin, ethinyl estradiol and levonorgestrel. Norethisteron and levonorgestrel are often used progestins in contraception pills.

Shortly after administration, ethinylestradiol is found mainly as sulphate conjugated (80 %) in plasma. Part of the compound is metabolised and occurs as 6α-hydroxy, 2-methoxy, 2-hydroxy-3-methylether and 16β-hydroxy derivatives as well as metabolites where the ethinyl group is missing. However, a large part is excreted in un-metabolised but conjugated form. 16.5 % of the dose can be refound in the urine and 9 % in faeces (*70;208;212*). This is in accordance with the assumptions of a total excretion of 26 % made by Johnson et al. (*72*). However, larger excretion factors have also been reported (*72*).

For the estimation of national emission, the value of 26 % ethinylestradiol excretion was used.

10.2 Release of estrogens

The total human excretion of estrogens in Denmark was calculated using the above excretion patterns and estimates of the demographic distribution of women and men in Denmark.

Table 10.10. Demographic figures for Denmark 2001(*75*)

Group	Number
Men (13 < age)	2,163,874
Menstruating women (12 < age < 51)	1,347,006
Living new borne	67,000 /year
Menstruating women not pregnant	1,296,756
Pregnant women (9/12 * 67,000)	50,250
Post menopause women (50 < age)	930,708
Post menopause women (50 < age< 56)	197,289
Post menopause women (55 < age< 61)	165,946
Post menopause women (60< age< 66)	131,179

Estimates of the natural excretion based on the demographic figures in Table 10.10. and on the individual excretion patterns
given in previous chapters are presented in Table 10.11.

Table 10.11. Total natural excretion of estrogens in Denmark 2001

	Men	Women			
g/24 h		Menstrual	Pregnant	Post menopausal	Total
Estriol	3.2	6.5	301	0.9	312

Estrone	8.4	10.8	30.2	3.7	53.1
Estradiol	3.5	4.7	13.0	2.1	23.3

In Denmark, 435,000 women are using oral contraceptives (*213*). The pills taken for 21 days in general contain 30-50 µg ethinylestradiol (*214*). This gives an average of 12.2 g ethinylestradiol per 24 h in Denmark which corresponds with data from the Danish Medicines Agency (*215*). With an estimated excretion of 26 %, this corresponds to the excretion shown in Table 10.12.

Table 10.12. Total excretion of ethinylestradiol in Denmark 2001

g/24 h	Total
Ethinylestradiol	3.2

In Denmark, 80 % of all women suffer from the menopausal symptoms. 25 % still have problems after 5 years and after 10 years, 15 % still have problems. Among these, some will have symptoms for the rest of their postmenopausal life (*214*). The amount of estradiol sold to relieve menopausal symptoms is stated to be 23 mill. DDD in year 2000 by The Danish Medicines Agency (*215*). The DDD (equal to the content of one pill, taken each day), range between 1 and 2 mg estradiol or estriol (*214*). An average of 1.5 mg is used for the estimations. This gives a total use of estradiol and estriol of 97 g per 24 h in Denmark and a total release via faeces and urine at 78 g of estriol, estradiol and metabolites per 24 hours. The distribution between different metabolites is presumably similar to that shown in Table 10.11.
If this distribution is used, the excretion from hormone therapy of post-menopausal women is as given in Table 10.13.

Table 10.13. Total excretion of estrogens from hormone therapy in Denmark 2001

g/24 h	Total
Estriol	27.6
Estrone	15.5
Estradiol	12.3
Other	20

The total excretion is given in Table 10.14.

Table 10.14. Total estimated excretion of estrogens in Denmark 2001

g/24 h	Total
Estriol	339.8
Estrone	68.6
Estradiol	35.7
Ethinylestradiol	3.2

10.2.1 Other sources

10.2.1.1 Phytoestrogens

Though many plants have been shown to contain estrogenically active compounds, only a few contain estradiol and estrone. Examples are liquorice, French bean, date palm, pomegranate and apple (*216*). The release of estrogens from these plants is not expected to be a major source of estrogens in waste water.

10.3 Alkylphenols

Alkylphenols (AP) consist of a phenol group and an alkane. The compounds may vary both in the relative position of the alkane group and in the length and branching of the alkane. Commercially available alkylphenols are generally mixtures of alkylphenols with different degrees of branching but with the same number of carbon atoms in the chain (*76*). The largest European producer Sasol offers amyl-, butyl-, cumyl-, dodecyl-, nonyl- and octylphenols for different applications (*217*). However, nonylphenol (NP) is the most commercially prevalent of the alkylphenol family, representing approximately 85 % of the alkylphenol market. Alkyphenols are mainly used in the production of alkylphenol ethoxylates (APEs), tris(nonylphenyl) phosphite (TNPP) and alkylphenol-formaldehyde condensation resins (*77*). However, unreacted alkylphenols can be used as plasticizers in plastics.

Table 10.15. The use of nonylphenol in the EU in 1997 (*76*)

	Amount	
	tonnes/year	[%]
Production of nonylphenolethoxylates	47000	60
Production of resins, plastics, stabilisers etc.	29000	37
Production of phenolic oximes	2500	3
Total	78500	100

10.3.1 Nonylphenolethoxylates

Nonylphenolethoxylates are relatively easily degraded to nonylphenol (*78*) and therefore, an important source of alkylphenols. In 1997, 47,000 tonnes of nonylphenol were used for producing 118,000 tonnes of nonylphenolethoxylates in the EU. To our knowledge, this production, performed by seven companies, takes place outside Denmark.

Nonylphenolethoxylates were in 1994 used for the following “EU use categories” in the EU.

Table 10.16. Use of nonylphenolethoxylates in the EU in 1994 (*76*)

Functional use	%
Cleaning and washing agents	44.7
Surface active ingredients	46.1
Foaming agents	2.8

Flotation agents	1.7
Cosmetics	1.5
Construction materials and additives	1.4
Dust binding agents	1.4
Intermediates	0.2
Pesticides agricultural (plant protection)	0.1
Others	0.1

10.3.1.1 Industrial and institutional cleaning

Nonylphenolethoxylates are used in laundries, for floor and surface cleaning in buildings, for car washing, as anti-static cleaners and for metal cleaning. Domestic use of nonylphenol-based cleaners should be virtually zero due to bans and agreements and the use for industrial cleaning should also decrease. The Danish EPA made a voluntary agreement with 'The Association of Danish Cosmetics, Toiletries, Soap and Detergent industries (SPT)' in 1987 concerning a reduction of the use of alkylphenolpolyethoxylates. The members of SPT cover 80 to 90% of the Danish market and an analysis carried out by the Danish EPA showed that only one out of 34 cleaning products contained nonylpolyethoxylate or octylpolyethoxylate.

The amount of nonylphenolethoxylate used for industrial and institutional cleaning in the EU was 23,000 tonnes in 1997 (*76*) and in Denmark, the amount used in 1995 was 1,066 tonnes (*218*). In view of the voluntary agreements, these numbers probably overestimate the present Danish use of alkylphenolpolyethoxylates which may then be estimated to be between 0 and 1,066 tonnes.

10.3.1.2 Textile auxiliaries

Nonylphenolethoxylates are used in scouring, fibre lubrication and dye levelling in the textile industry. The main use is in wool scouring (*76*), which is not considered important in Denmark where wool production is small. The total amount of alkylphenol ethoxylates used in textile auxiliaries was 8,000 tonnes in 1997 (*76*).

10.3.1.3 Leather auxiliaries

Nonylphenolethoxylates may be used for degreasing in the preparation of hides. At present, only very little tanning is taking place in Denmark as Elmo Svendborg is the only functioning tannery. However, a very large tannery is planned on the island of Funen. Within the EU, 3,137 tonnes/year of nonylphenolethoxylates are estimated to be used in leather auxiliaries (*76*).

10.3.1.4 Agriculture

Nonylphenolethoxylates are used as wetting agents in agrochemical formulations. This decreases the necessary amount of active ingredient. It is also used in tit dips for cows and sheep. In Denmark, the amount used for pesticides in 1995 was 175 tonnes (*218*), but an agreement was made with the Danish pesticide producers in 1995 to phase out the use of alkylphenolpolyethoxylates in pesticides. Presently, no pesticides on the market contain alkylpolyethoxylates.

10.3.1.5 Emulsion polymers

Nonylphenolethoxylates are added to acrylic esters used for coatings, adhesived and fibre bonding. They act as dispersants and aid in stabilising the formulation. Nonylphenolethoxylates are also thought to be present in the polymerisation reactions used to make polymer solutions for waste water treatment (*76*). The amount used for this purpose is not known.

10.3.1.6 Paints, adhesives and sealants
Nonylphenolethoxylates are used in the preparation of paints, adhesive and sealant resins. Especially in water-based products (*219*). In the EU approximately 4,000 tonnes of nonylphenolethoxylate were used for paints and lacquers in 1997 (*76*) while, in Denmark, 63 tonnes were used for paints and lacquers and 77 tonnes for fillers in 1995 (*218*).

10.3.1.7 Pulp and paper
Nonylphenolethoxylates may be used in the pulp and paper industry in the wetting of pulp fibres, as anti-foaming agents and as retention aids. The total use of nonylphenolethoxylates in this industry in the EU is estimated to be 800 tonnes/year (*76*).

10.3.1.8 Metal industry
Nonylphenolethoxylates are used in the cleaning of metal surfaces (iron and steel manufacture), for metal phosphating electronics cleaning and for cleaning of metal product prior to storage. It is also used in cutting and drilling oils (*76*).

10.3.1.9 Photographic industry
Nonylphenolethoxylates are used as wetting agents in the developing of photographic film, mainly amateur use. The amount used in Europe for this purpose is estimated to be 93 tonnes/year (*76*).

10.3.1.10 Lubricants and motor oil industry
Nonylphenolethoxylates may be added to motor oil and fuels to make engines meet emission demands. They lubricate and clean engines. In Denmark, products for cooling and lubrication contained 33 tonnes of nonylphenol or nonylphenolethoxylate in 1995 (*218*).

10.3.1.11 Insulation
In Denmark, 77 tonnes/year were used as additives for insulation materials in 1995 (*218*). This is assumed to be partly for refrigerator linings.

10.3.2 Other derivatives of alkylphenols

Other important derivatives besides alkylphenolethoxylates are alkylphenol phosphites and nonylphenol-formaldehyde condensation resins (*76;77*). In the EU, the amount of nonylphenol used for production of these derivatives was 29,000 tonnes in 1997 (*76*).

10.3.2.1 Alkylphenol phosphites
Alkylphenol phosphites can be used as UV stabilisers in plastics. 4,000 tonnes nonylphenol were used for production of tri(4-nonylphenol)phosphite in 1997 in the EU (*76*).

10.3.2.2 Nonylphenol-formaldehyde condensation resins
The main use of nonylphenol in the plastics industry is as a monomer in the production of phenol/formaldehyde resins, which are used as adhesives and tackifiers in the rubber industry, as paper coating resins, as resins for printing inks and as resins for contact adhesives and coatings. According to the EU, 22,500 tonnes of nonylphenol were used for nonylphenol-formaldehyde resins in 1997, at 25 sites in the EU (*76*).

10.3.2.3 Phenolic oximes
Within the EU, 2,500 tonnes of nonylphenol were used for the production of phenolic oximes in 1997. This took place at one site outside Denmark (*76*). Phenolic oximes are used in extraction and purification of copper from ore, a process that is not performed in Denmark.

10.3.2.4 Nonylphenol amine salts
In the EU, 1,500 tonnes of nonylphenol were used for the production of nonylphenol amine salts. This production is not known to take place in Denmark. Nonylphenol amine salts are used as curing agents or accelerators for epoxy resins. With curing, the nonylphenol is covalently bound inside the epoxy matrix (*76*). In Denmark, hardeners containing 66 tonnes of nonylphenol were used in 1995 (*218*).

10.3.2.5 Miscellaneous uses
Apart from the major uses outlined above, a number of miscellaneous uses of nonylphenol derivatives are known: Carbonless copy ink, latex manufacture, and packaging materials (*77*). The derivatives and amounts used for these purposes were not identified in the present assessment. They are, however, expected to be of minor importance.

10.4 Release of alkylphenols

Nonylphenol is produced at four sites within the EU (*76*), of which none seems to be in Denmark. Thus, it is believed that there is no release from production in Denmark. Likewise there seems to be no large production of nonylphenolethoxylates or other derivatives and consequently no associated release, in Denmark. Therefore, the release of nonylphenol is expected to be mainly from processing/formulation and use.

10.4.1 Release from processing/formulation

Though neither alkylphenols nor derivatives are produced in Denmark, they may be added to formulations in Denmark. For nonylphenolethoxylates, an emission factor of 0.003 to waste water is used in the EU risk assessment, which is in line with industry reports (*76*). This leads to a total release of 216 tonnes of nonylphenolethoxylates/year, from formulation, in the EU. In Denmark, a release factor of 0.003 from formulation of cleaning products and tensides with nonylphenolethoxylates corresponds to a release of 4.4 tonnes nonylphenolethoxylates/year to waste water.

10.4.2 Release from use

The major release of nonylphenol from use is expected to be from nonylphenolethoxylate used as a detergent.

10.4.2.1 Release from use of nonylphenolethoxylate
Industrial and institutional cleaning:
The wastewater release factor for institutional and industrial cleaning is estimated to be 0.9 (*76*). With a yearly use of 23,000 tonnes, this results in a release of 20,700 tonnes nonylphenolethoxylate/year in the EU and between 0 and 959 tonnes in Denmark.

Car wash:
The release of nonylphenol from car wash in Denmark to waste water has been estimated to be around 891 kg/year (*220*)

Pesticide:
The usual dose applied with pesticide used to be 50-200 g nonylphenolethoxylate/ha (*76*). It is assumed that all use of nonylphenolethoxylate in pesticides in Denmark has ceased.

Veterinary tit dips:
There may be some release from the use of nonylphenolethoxylates in tit dips for cows. However, this is not expected to reach the sewage system but rather to be spread on agricultural land with manure.

Leather processing:
The EU release factor for the use of nonylphenolethoxylates in leather processing, to waste water is set to 0.9. Approx. 2,700 tonnes of NPEO are thus released yearly from the leather processing industry. The average release from one large site is estimated to be 2.7 tonnes/year (*76*).

Metal industry:
In the EU the release of nonylphenolethoxylates from the metal processing industry is estimated to be 632 tonnes/year to waste water (*76*).

Photographic industry:
Nonylphenolethoxylates used as wetting agents may be released to waste water. With the EU emission factor of 0.8 to waste water, this gives an emission of 74 tonnes/year in the EU (*76*).

Pulp paper an board industry:
The paper industry is very small in Denmark, why only limited releases from this area can be expected. In the EU, 800 tonnes/year of nonylphenolethoxylates is estimated to be released from the use in this industry (*76*).

Textile processing industry:
The factor for emission of nonylphenolethoxylate, used in textile processing, to waste water, is set as 0.85 by the EU. This leads to an emission of approx. 4,000 tonnes in 1997. The majority is used for initial wool processing, which is not commonly performed in Denmark.

Paints, lacquers and varnishes industry:
During paint manufacture, release of nonylphenolethoxylate may take place. Estimated emisson factors of 0.005 for emission to waste water have been given by the industry. This leads to an emission of 20 tonnes/year for EU and 0.3 tonnes for Denmark.

Cosmetics:
It is estimated that 50 % of the nonylphenolethoxylate used in cosmetics are released to waste water. The remainder is expected to be disposed of as solid waste. This gives a release of 10 tonnes/year to waste water in Denmark.

Insulation materials:
In Denmark, some use of nonylphenolethoxylate and nonylphenol for insulation (polyurethan) in Denmark has been noted. The loss from this process is not known.

10.4.2.2 Release from use of other alkyphenols

Release from the use of other alkyphenols, mainly in the plastics industry is expected to be low. For example, the loss from use of hardeners is expected to be low. However, this release has not been quantified.

10.4.3 Release from disposal

Most alkylphenol (nonylphenol) is used in products such as detergent formulations, which are not disposed of but emitted to waste water during use. However, alkylphenol incorporated in products such as plastic, paints etc. may be disposed of to landfill or incineration. Alkylphenol will disappear by incineration. No knowledge on the fate in waste deposits is available.

A summary of estimated releases is given in Table 10.17. Where figures for Danish production have been available, these have been used. Since the former use of alkylphenolpolyethoxylates in cleaning products was the largest contributor to the release to the environment, the present release depends strongly on the success of the voluntary agreement between the Danish EPA and 'The Association of Danish Cosmetics, Toiletries, Soap and Detergent industries (SPT)'.

Table 10.17. Summary of Nonylphenolethoxylate releases to waste water (tonnes/year)

	EU	Estimation factor	DK
Formulation of detergents and tensides	-	-	4.42
Cleaning			0 - 959[a]
Car wash	-	-	0.891
Leather	2700	0.1	3.90
Metal	632	1	9.13
Photographic	74	1	1.07
Pulp and paper	800	0.1	1.16
Textile	4000	0.1	5.78
Paint			0.32
Cosmetics			10
Total			37 - 996

a: Depending on the success of the voluntary agreement

Nonylphenol accounts for 85 % of the alkylphenols sold. The main part of the remaining 15 % is assumed to be octylphenol used in the same way as nonylphenol. The released amounts of alkylphenols could thus probably be enlarged by a factor 1/0.85.

10.5 Bisphenol A

Bisphenol A is used for many purposes in modern society. The major consumption is related to the use of bisphenol A as a chemical building block in the production of polycarbonate plastic and epoxy resins (*79*). However, bisphenol A is also used for numerous other purposes, as shown in Table 10.18. Formerly, bisphenol A was used as an inactive ingredient in pesticides but this use has ceased (*221*).

Four companies within the EU produce bisphenol A but there seems to be no such production in Denmark. Total production within the EU is around 700,000 tonnes/year and consumption is around 690,000 tonnes a year (Table 10.18).

Table 10.18. Use in Europe (*221*)

Use in Europe	Amount	
	Tonnes/year	Percentage
Polycarbonate production	486,880	71.1
Epoxy resin production	171,095	25.0
Phenoplast resins	8,800	1.3
Unsaturated polyester resin production	3,000	0.4
Can coating manufacture	2,460	0.4
PVC production and processing	2,250	0.3
Alkyloxylated bisphenol A manufacture	2,020	0.3
Thermal paper manufatcture	1,400	0.2
Polyols/polyurethane manufacture	950	0.1
Modified polyamide production	150	<0.1
Tyre manufacture	110	<0.1
Brake fluid	45	<0.1
Minor uses	5,990	0.9
EU consumption	684,650	

10.5.1 Polycarbonate

Polycarbonate is produced by a polymerisation process involving bisphenol A. This process is not takin place in Denmark (*222*), but at five sites in the EU. Although the bisphenol A in polycarbonate in general is polymerized, there are small amounts of free bisphenol A in the final polycarbonate, between <10 mg/kg and 50 mg/kg (*221*). Polycarbonate is used for compact disks, reusable bottles, food contact containers, multi-wall sheets for construction, injection moulded structural parts for the electronics and automotive industry, impact-resistant glazing, street-light globes and household appliance parts (*79;221*).

10.5.2 Epoxy resins

Bisphenol A is used for making epoxy resins at 8 sites in the EU, none of them in Denmark (*222*). The epoxy is used for protective coatings, food- and beverage-can linings, structural composites, electrical laminates, electrical applications and adhesives (*79;221*). Some epoxy resin adhesives for domestic use, available on the Danish market, contain bisphenol A (*223*). Bisphenol A may be ethoxylated for use in special epoxy resins.

10.5.3 Phenoplast resins

Phenoplast resins (tradename bakelite®) are hard brittle plastic materials used for electrical appliances, kitchen tables and car engine parts. There is no phenolplast production in Denmark (*222*).

10.5.4 Can coating manufacture

Can coating is produced by reacting an epoxy resin with bisphenol A. According to the EU, only 5 companies in Europe make can coatings containing bisphenol A (*221*). A Danish company, Glud og Marstrand A/S, performs can coating with bisphenol A epoxy in Denmark.

10.5.5 Thermal paper

Bisphenol A is used in the coating of thermal paper, in which it works as a developing agent. Not totally reacted paper may contain significant amounts of residual bisphenol A. Thermal paper is used for cash registers, thermal

faxes and printers, and for other machines in hospitals, laboratories etc. (*221*). Thermal paper is not produced in Denmark.

10.5.6 PVC

Bisphenol A is used in the PVC industry as a polymerisation inhibitor and as an anti-oxidant. The use as a polymerisation inhibitor does not take place in Denmark as there is no PVC raw material production in Denmark (*224*). In the rest of the EU, bisphenol A used for this purpose should be voluntarily phased out since 2001 (*221*).

Bisphenol A is used as an anti-oxidant in the processing of PVC, in the production of plasticisers and in the preparation of additive packages for PVC processing. The production of plasticisers and additive packages is not expected to take place in Denmark, but processing of PVC, involving plasticisers and antioxidants, does.

10.5.7 Other uses

Bisphenol A is used in the production of polyols, which are used in the production of polyurethane and in the production of unsaturated polyester resin. None of these productions takes place in Denmark (*222*). In Denmark, bisphenol polyester resin is used in toners for printers and photocopiers, and polyurethane is used for insulation (*225*).

10.5.8 Brake fluid

Bisphenol A is added to brake fluid as an anti-oxidant at one site in the EU (*221*) but not in Denmark.

10.5.9 Tyre manufacture

Bisphenol A is used as an antioxidant in the process of tyre manufacturing, but bisphenol A is not expected to be present in the finished tyre in significant concentrations (*221*). No tyre production takes place in Denmark.

10.5.10 Fire retardant

Bisphenol A was previously used for the production of tetra-bromobisphenol, which has been used as a fire retardant, but this production is probably not relevant any more within the EU (*221*).

10.6 Release of bisphenol A

There is no release to the environment from the production of bisphenol A in Denmark, as there is no production. Likewise there is no production of polycarbonate, epoxy resin or other plastic raw materials and thus no related release.

In Denmark, releases of bisphenol are thus expected to arise from processing, use and disposal of bisphenol A-containing materials.

10.6.1 Release from processing

Studies have shown that the release of unreacted monomers during processing of polycarbonate in general is very small (undetectable). However, if the correct procedures are not followed, released amounts may be larger (*221*).

There may be some release of bisphenol A in relation to the use as an anti-oxidant and plasticiser in PVC processing in Denmark. In the EU risk assessment report, the use of 3 tonnes per year as anti-oxidant, typical amount for one generic PVC processing site, is estimated to lead to the release of 6.42 kg/year to waste water (*221*). In the EU, the total loss to waste water, from the use as PVC antioxidant, is estimated to be 1,070 kg/year. The use of bisphenol A as a plasticiser in the processing of PVC may lead to releases to waste water in the order of 140 kg/year for EU (*221*).

In the EU risk assessment (*221*), no release of bisphenol A from can coating to surface or waste water has been encountered at five sites. Waste is disposed of by incineration (*221*). Thus, no significant emission to water from the Danish can coating industry is expected.

10.6.2 Release during use

Studies have shown very low migration of bisphenol A from polycarbonate into foodstuffs (*221*). The release to the environment through foodstuffs is thus considered negligible. The total release from washing of polycarbonate bottles, reused for beverages, is estimated to be very small, maximally 1.6 kg/year in the EU (*221*). Likewise, the release from installed multi-wall polycarbonate sheets is very small, estimated to be 1.3 kg/year in the EU and considered insignificant (*221*).

The release of bisphenol A from epoxy resins is low. The content of unreacted bisphenol A in uncured epoxy resins is lower than 1,000 ppm and the content is reduced when the resin is cured. The release of bisphenol A from can coatings to food is also very small (*221*).

The release of bisphenol A from phenoplast is expected to be very small (*221*). The production and use of phenoplast are much smaller than that of polycarbonate and release can be expected to be much lower as e.g. no washing occurs.

Plasticisers containing bisphenol A are mainly used in PVC for roofing and cables, and some release from these items can be expected. Likewise, some release can be expected from PVC products containing bisphenol A for other purposes. These releases are not directly to waste water but in Denmark, most surface run off from industrial and residential areas is still discharged into the sewage system. The emissions from the use of PVC products are given in Table 10.19.

Table 10.19. Emissions from the use of PVC products in the EU (*221*)

	Air	Surface water	Soil
	tonnes/year		
Anti oxidant from PVC processing	7. 5	11.1	11.1
Additive packages	7.5	11.1	11.1
Anti oxidant in plasticisers	0.6	0.5	0.5
Total	15.6	22.7	22.7

Production of brake fluid is not known in Denmark and releases from use are expected to be small. Bisphenol A is destroyed in use and waste is in general disposed of as chemical waste (*221*).

No tyre manufacturing takes place in Denmark and no loss of bisphenol A is expected during use (*221*).

10.6.3 Release from disposal

After end of use, the plastic products containing small amounts of bisphenol A can be disposed of to landfills or incinerated. By incineration, any free bisphenol A is effectively destroyed.

Considerable leaching of bisphenol A from hazardous waste landfills has been measured in Japan (*226*) and in Germany (*221*). Conceivably, it may originate from deposited plastics (polycarbonate, PVC, epoxy resins) (*226*). Experiments in which pieces of plastic have been soaked in water for a couple of weeks, have shown that leaching from synthetic leather and PVC cords can be high, up to 139 µg/g plastic was measured (*227*). However, thermal paper could also be an important source. It has not been possible to estimate the release from landfills here.

10.6.4 Release from recycling

There may be a significant loss of bisphenol A to water from recycling of thermal paper. In the deinking process bisphenol A is released to water. Even though the deinking waste water is treated in a sewage treatment plant at the paper mill, a large emission may be expected according to the EU risk assessment (*221*). Rigol et al. have reported concentrations of up to 100 µg/l in recycling process waters (*228*) and, in Japan, bisphenol A concentrations ranging from 8 to 370 µg/l have been measured in the final effluents from paper recycling plants (*229*). In Denmark, paper is reused by the companies Brdr. Hartmann A/S (Tønder), Dalum paper mill (Odense), SCA Djursland (corrugated cardboard) and Skjern papirfabrik (*230;231*). However, thermal paper is only expected to constitute a small part of the paper reused at these sites. In the EU, a yearly release to waste water, after pre-treatment at paper mill, of 340 tonnes has been estimated (*221*). Fürhacker et al. found that paper industry was by far the largest contributor to bisphenol A in sewage water led to a STP in Austria (*232*). Whether the source was thermal paper was not discussed. It should be noted that bisphenol A may be transformed to the more chlorinated bisphenol A if the pulp is bleached with chlorine (*229*).

The releases relevant for Denmark have been extrapolated from European to Danish amounts below by means of the relative size of the populations and by application of an estimation factor expressing the difference in European and Danish conditions. For example, a relatively smaller amount of thermal paper

is expected to be reused in Denmark than in Europe in general and consequently an estimation factor less than 1 was chosen. The relationship between populations is 5.35 mill (DK) to 370 mill (EU).

It must be noted that these are very crude estimates, which should be substantiated by thorough substance flow investigations.

Table 10.20. Summary of bisphenol A releases to wastewater (tonnes/year)

	EU	Estimation factor	DK
PVC processing	1.2	1	0.017
PVC use	15.6	1	0.256
Paper reuse	340	0.1	0.492
Total	356.8		0.735

11 Fate in municipal sewage treatment plants

Influents, effluents and sewage sludge of STPs have been monitored in several countries, e.g. Germany, Switzerland, Italy, the U.K., Spain, the U.S., Canada, Japan, and Denmark, with the purpose to study the occurrence of estrogens as well as xenoestrogens. A considerable amount of these studies have only included analysis of effluents, which is certain to give a picture of the concentration levels and the possible amount of substance discharged to the aquatic environment. However, more detailed monitoring of the material streams around each single process in a STP is needed for assessment of the fate of a substance in the treatment plant.

Results are presented in the scientific literature from examinations of parallelly collected influent and effluent samples from STPs and some times also samples of sewage sludge. However, it should be born in mind that variable procedures of sampling have been used from one study to another and, in some cases, even within the same study. Also the applied analytical methods including their limits of detection and determination vary between different studies. These facts limit the possibilities of an exhaustive evaluation of the fate of the compounds in STPs and of an assessment of the effect of different treatment processes.

A review of the effluent concentrations of the substances of concern is presented in Chapter 5. This chapter will primarily focus on investigations, which allow an assessment of the behaviour of the substances in a STP, i.e. studies which, as a minimum, have included simultaneous monitoring of either influent and final effluent from a STP or in- and outlet of specific treatment processes within a STP.

11.1 Estrogens

11.1.1 Biodegradation in sludge of STPs

The majority of natural estrogens and contraceptive compounds are excreted from humans as a variety of inactive glucuronide or sulfonide conjugates as described in Chapter 10. It has been questioned whether these inactive conjugates are cleaved in the STP and perhaps already in the raw sewage and thereby released to the environment as active estrogens. The detection of several unconjugated estrogens like estradiol, estrone, estriol and ethinylestradiol, in the effluents from STPs supports this hypothesis.

The biodegradability of (4-^{14}C)-estradiol has been examined according to the principle in semicontinous activated sludge test (SCAS test) described in the OECD Guideline No. 302A and the "Activated sludge biodegradability simulations test", Environmental Project No 337 (*83*). The test simulated an activated sludge basin at municipal STPs. The biodegradation was investigated under both aerobic and anoxic conditions during 120 h at 15 °C. The experiments were made in triplicates. Reactors of one litre were

inoculated with activated sludge from Måløv STPs in Denmark achieving a final concentration at 5 g suspended solid (SS)/l. Filtrated supernatant from settled activated sludge was used as substrate at the initiation of the test and later a peptone medium. This medium was supplemented with nitrate in the anoxic experiments. The initial concentration of estradiol was approx. 20 µg/l. The total and dissolved amounts of ^{14}C in the reactors were analyzed by liquid scintillation. Determination of the mineralisation rates of (4-^{14}C)-estradiol under aerobic condition showed a first order rate constant for total and dissolved estradiol of 0.031 ± 0.003 l/d/g SS and 0.052 ± 0.003 l/d/g SS, respectively, when the concentration was less than 2.5 µg/l. No significant degradation of (4-^{14}C)-estradiol was observed in the anoxic test system. Average sludge distribution coefficients K_d for ^{14}C labelled compounds in the aerobic and anoxic test system with Måløv sludge were estimated at 0.25 ± 0.04 l/g SS and 0.96 ± 0.10 l/g SS, respectively (*83*).

The aerobic transformation of natural estrogens, contraceptives and estradiol glucuronides has recently been studied in a batch experiment using suspensions of activated sludge at 0.26 g SS/l as inoculum (*80*). The experiment showed that the glucuronide conjungates of estradiol were de-conjugates relatively fast. When spiking the sludge slurry with approx. 1 mg/l of estradiol glucuronides, both estradiol and estrone were detected after only 15 min. Approx. 70 % of the conjungated estradiol were found in the oxidized form, estrone, at the maximum concentration after 20-30 h. However, estradiol was still detected after 28 h indicating that the cleavage of the glucuronide was not completed. That glucuronides are cleaved in slurries of activated sludge was confirmed in another experiment with a spiking level at 1 µg/l and in this case with even higher turnover rates. Furthermore, it was found that, when in contact with activated sludge, the estradiol was oxidized into estrone that was further eliminated. The contraceptive ethinylestradiol was principally persistent under the condition used in the experiment.

Furthermore, the transformation of the inactive excreted glucuronides into the active estrogens in sewage have been documented by Panter et al. (*81*). They suggested that estradiol-3-glucuronide were converted into active estrogens after having been inoculated with activated sludge. The suggestion was based on studies of in-line degradation systems allowing fish to be exposed before and after biodegradation of conjugated estradiol. The studies demonstrated that solutions after passing through a degradation system with activated sludge microorganisms were highly estrogenic.

Layton et al. (*84*) have performed a series of biodegradation studies with steroidal hormones in laboratory assays inoculated with biosolids obtained in the period 1998-2000 from aeration basins of four different STPs in the U.S.A. The initial concentrations of the estrogenic hormones, 17β-estradiol and 17α-ethinylestradiol, were 58 and 72 µg/l, respectively. The steroids were ^{14}C-labelled on the C-4 carbon of the steroid backbone. Therefore, release of ^{14}C-CO_2 from these compounds would denote ring cleavage and concomitant inactivating of the steroid molecule. In biosolids taken from the aeration basin of a municipal STP, 84 % of ^{14}C-estradiol and 85% of ^{14}C-estrone were mineralized to ^{14}C-CO_2 within 24 hours of incubation. The mineralisation of the same compounds in biosolids from an industrial STP was considerably lower (4%). This confirmed the importance of an adapted microbial population in the biological removal of estrogens. There were no statistical differences in the mineralisation in biosolids between municipal STPs with

different operations parameters, e.g. different percentage of BOD removal and percentage of suspended solids removal.

Investigation of the mineralisation of ethinylestradiol confirmed the results of Ternes et al. (*80*). The mineralisation rate of ethinylestradiol was low compared to the rate of estradiol resulting in a removal of 20 % ^{14}C-ethinylestradiol versus 75 % of ^{14}C-estradiol in the same type of biosolid after 24 h of incubation (*84*). Determination of the first-order rate constant k for removal by mineralisation or removal from the aqueous phase of the ^{14}C-steroids shows no significant differences for ethinylestradiol at temperatures differing by 10-15°C. The observed differences of k values for estradiol at different temperatures were statistically significant. However, it was noted by Layton et al. (*84*) that the initial mineralisation rates of estradiol at 5-10 °C were 200 ng/l in a minute suggesting that, even at low temperatures, estradiol is rapidly removed. Sorption was not found to be the rate-limiting step at the tested concentrations of estradiol. The obtained rate constants for mineralisation of estradiol were 0.0029 ± 0.0002 min^{-1} and 0.0042 ± 0.0002 min^{-1} at 5-10°C and 22-25°C, respectively. The rate constant for mineralisation of ethinylestradiol were 0.0001 ± 0.0000 min^{-1} and 0.0002 ± 0.0000 min^{-1} at 5-10°C and 22-25°C, respectively.

Degradation of [^{3}H]-ethinylestradiol has been studied in a batch experiment with activated sludge grown in a laboratory continuously fed activated sludge (CAS) reactor. The reactor was fed with a mineral medium allowing the growth of a highly active nitrifying sludge (50 mg NH_4^+/g dry weight/h). Ethinylestradiol (50 µg/l) added to sludge from this reactor together with hydrazine was degraded or transformed to hydrophilic products within six days of incubation at 20°C. The degradation products were not identified. In a similar experiment in activated sludge with an ammonium oxidation rate of only 1 mg NH_4^+/g dry weight/h no detectable degradation of ethinylestradiol was observed (*233*).

It should be noticed that the steroid estrogens were the sole added carbon source besides the naturally occuring carbon in the activated wastewater sludge in the degradation studies performed by Ternes et al. *(80)*, Layton et al. *(84)* and Vader et al. *(233)*. The situation is quite different in a full-scale STP, in which the influent consists of a broad spectrum of different carbon sources. It is, therefore, difficult to assess the situation in a real STP based on the degradation rates obtained in the present lab-scale studies.

The possible initial degradation steps of estradiol glucuronides based on the results of the studies presented above are shown in Figure 11.1

estradiol-glucuronide ⟶ estradiol ⟶

Figure 11.1. Initial degradation steps of estradiol-glucuronides in aerated activated sludge

11.1.2 Sorption to sludge particles

Upon examination of log P_{ow} for the estrogens of concern, one will expect that ethinylestradiol (log P_{ow} = 4 (*234*)) and estradiol (log P_{ow} = 4.01 (Pomona 1987)) are likely to be sorbed to sludge and possibly also estrone (log P_{ow} = 2.1-3.5 (*235*), (*234*)). Estriol with a log P_{ow} at 2.7 (*234*) is, however, considered to be less hydrophobic and binding to sludge would be more unlikely to dominate the fate of estriol.

11.1.3 Fate in STPs

There are no available data on extensive investigations of the fate of estrogens in STPs covering the different process step throughout the STP and only few studies include analysis of samples from internal streams of STPs. Shore et al. (*236*) studied samples of raw sewage, reaction fluid after aerobic treatment, the supernatant of activated sludge after digestion and the final effluent taken from a STP in Israel in 1991. Analysis of estradiol using radioimmunoassay showed concentrations of estrogens in raw sewage at 48-141 ng/l and a relative removal of estrogens in the water phase of 20-88 %.

A study of 27 STPs was performed in Japan during 1998-1999. The study was made three times during the season, one in the summer, one in the autumn, and one in the winter (*89*). The STPs used mostly an activated sludge process. Some of the plants had nitrogen and phosphate removal and the plants were, furthermore, equipped with different disinfection treatments. Samples were taken of the influent of the STPs, the influent of the primary sedimentation tank (i.e. after mixing with reject water from the sludge treatment process), the effluent after primary sedimentation and the effluent after final sedimentation before and after desinfection. Analysis of 17β-estradiol in the samples showed median removal efficiency in the water phase, i.e. from influent to final effluent at 69 % and 64 % in the autumn and the winter studies, respectively. The fate of 17β-estradiol in the sewage treatment process was illustrated with an example in the work of Nasu et al. (*89*). The median values of these data are shown in Appendix A. The data indicate that there was a minor increase in the influent concentration of 17β-estradiol after mixing with reject water and after the primary sedimentation. However, the differences do not seem to be statistically significant. The biological treatment process and the final sedimentation resulted in a distinct reduction of the 17β-estradiol concentration, while no further reduction was observed after the disinfection steps.

Ternes et al. (*86*) sampled corresponding influent and effluent samples of a German and a Brazilian municipal STP in 1997. The sampling at the German STP, furthermore, included samples taken from the effluent from the primary sedimentation tank. The Brazilian STP operated an aeration tank and a trickling filter (biological filter) in parallel. Effluent samples were collected from both of these biological treatment processes. The analyses of the samples included estrone, 17β-estradiol, 16α-hydroestrone (only German STP) and 17α-ethinylestradiol. The investigation of the German plant showed a minor elevation of the loads of estrogens and especially of estrone after the primary sedimentation (Appendix A). Ternes et al. (*86*) suggested that despite the statistically insignificant differences, this increase could be caused by a cleavage of conjungates like glucuronides, which are the principal excreted metabolites, during the STP process.

The investigation of the Brazilian plant showed that there were distinct differences between the reduction rate of the estrogens in the aeration tank and that of the trickling filter (Appendix A). The highest removal efficiency of the estrogens was found in the aeration tank, where removal was in the range of 78-99.9 % whereas the removal efficiency in the trickling filter was 64-92 %. The removal of 17β-estradiol (92-99.9 %) was higher than that of estrone (67-83 %) and 17α-ethinylestradiol (64-78 %) in both treatment processes.

The removal efficiencies in the German plant were remarkably lower than in the Brazilian plant. It was for instance observed that only 64 % of 17β-estradiol and ~ 0 % of the 17α-ethinylestradiol were removed during the overall treatment process. The low temperature in the German sampling period of –2 °C in average compared to above 20 °C in Brazil may have caused this difference.

Lee & Peart (*85*), Ternes et al. (*86*), Baronti et al. (*87*), Johnson et al. (*88*) and Nasu et al. (*89*) have all reported concentrations of estrogens in samples taken from either the influent or the effluent of primary sedimentation tanks and the final effluent from different STPs. The five investigations cover examination of 15 different STPs in total (Appendix A). There are no significant differences in the influent concentrations for the five series of investigations taking into account the use of different sampling techniques and analytical methods. They are all within the same concentration range for the four estrogens of concern according to the summarized data in Table 11.1. The result of the analyses of the corresponding effluent samples shows that the concentrations of all the estrogens are reduced during the treatment process in the STPs (

Table 11.2.). The removal of 17β-estradiol
and estriol are generally more extensive than the removal of estrone and 17α-ethinylestradiol. This is in accordance with the finding in the laboratory studies of the degradation of 17β-estradiol, estrone and ethinylestradiol of Ternes et al. (*80*) and Layton et al. (*84*) described above (section 11.1.1). The mean removal efficiencies based on the results from the examination of thirty parallel influent and effluent samples from five STPs in the extensive study of Baronti et al. (*87*) were following:

- Estrone % removal: 61 ± 38
- Estradiol % removal: 87 ± 9
- Estriol % removal: 96 ± 6
- Ethinylestradiol % removal: 85 ± 14

Table 11.1. Concentration ranges of estrogens in influent of STPs. Conjugates as e.g. glucuronides were not included in the analyses.

Substance	Unit	Lee & Peart (*85*) [3]	Ternes et al. (*80*)	Baronti et al. (*87*)	Johnson et al. [2] (*88*)	Nasu et al. (*89*)
Estrone	(ng/l)	41-75	27; 40	25-90 (132)[1]	11-87 (140)[1]	
17β-estradiol	(ng/l)	<5-15	15; 21	4.0-25	11-48	20-94
Estriol	(ng/l)	158-250		25-188		
17α-ethinylestradiol	(ng/l)		1.2; 6	0.4-13	<0.2-8.8	

1: a single measurement
2: data from three STPs in the Netherlands
3. data from one STP in Canada

Table 11.2. Concentration ranges of estrogens in effluents of the same STPs as in Table 11.1. Conjugates as e.g. glucuronides were not included in the analyses.

Substance	Unit	Lee & Peart (*85*) [2]	Ternes et al. (*80*)	Baronti et al. (*87*)	Johnson et al. [1] (*88*)	Nasu et al. (*89*)
Estrone	(ng/l)	14-18	6.8-23	2.5-82	2.1-47	
17β-estradiol	(ng/l)	<5	0.2-5.4	0.35-3.5	<0.6-12	<0.2-55
Estriol	(ng/l)	30-37		0.43-18		
17α-ethinylestradiol	(ng/l)		1.3-2.7	<0.3-1.7	<0.2-<1.8	

1: data from three STPs in the Netherlands
2. data from one STP in Canada

The importance of conjugated estrogens as a potential pool of estrogenically active substances has been widely discussed (e.g., Belfroid et al. (*237*), Panter et al. (*81*), Ternes et al. (*80*), Johnson & Sumpter (*95*). It has been shown that estradiol-glucuronid conjugates are very easily converted to free active estrogens by microorganisms from activated sludge (Panter et al. (*81*), Ternes et al. (*80*)). Furthermore, Johnson et al. (*88*) found that the deconjugated estrogens detected in influent were close to the expected total based on excretion values (*95*). During the examination of effluent from five STPs in the Netherlands, Belfroid et al. (*237*) found concentrations of glucuronids above the detection limit (estrone glucuronides) in the effluent of two municipal STPs (Appendix A). This let to the hypothesis that the hormone glucuronides are degraded or transformed back into hormones in the STPs. Recently, Johnson & Sumpter (*95*) have suggested that the deconjugation may already occur in the sewage system before entering into the STPs. However, this suggestion is not supported by the results obtained by Adler et al. (*82*). They examined the content of both un-conjungated and conjungated estrogens in raw sewage and sewage effluent from STPs in Germany and found that the conjungates contributed with up to 50 % of the total steroid concentration in raw sewage. A summary of the results is given in Table 11.3.

Table 11.3. The median of the concentration of un-conjungated and total (un- and conjungated) estrogens in influent and effluent of German STPs (*82*).

Substance	Unit	Influent		Effluent	
		total	Un-conjungated	Total	Un-conjungated
Estrone	(ng/l)	13	5.5	8	2.5
17β-estradiol	(ng/l)	3	1.5	0.8	0.2
17α-ethinylestradiol	(ng/l)	9.5	7	0.5	0.3

11.2 Alkylphenols

11.2.1 Biodegradation in sludge of STPs

Alkylphenols (AP) in STPs are mainly a result of the biodegradation of alkylphenol polyethoxylates (APEO). Descriptions of the aerobic and anaerobic biotransformation pathways of APEOs show that the degradation is initiated by sequentially cleaving ethoxylate units (e.g. Ahel et al. (*93*), and the review by Ying et al. (*238*). Under aerobic conditions, the resulting products are alkylphenol (AP), mono- and diethoxylates (AP1EO, AP2EO), and the more hydrophilic mono- and dicarboxylates (AP1EC, AP2EC) according to

Ahel et al. (*93*). The transformation under anaerobic conditions results in the production of AP1EO, AP2EO and finally AP.

Investigation of the biodegradation of nonylphenol (NP) in lab-scale semi-continuous activated sludge (SCAS) reactors has shown that it is degradable under aerobic conditions and that its degradation is temperature-dependent (*239*). The influent solution to the reactors consisted of either a synthetic medium or effluent from a full-scale STP. Removal efficiencies of more than 99 % were obtained in 1.2 L SCAS reactors operated for 47 days at a temperature of 28 °C and given doses of approx. 5 mg NP three times a week. Mass balance over the reactors indicated that the added NP was biologically degraded according to Tanghe et al. (*239*). A subsequent lowering of the temperature from 28 °C to 10-15 °C and an increase of the loading rate from 1 to 2 g COD/l/d resulted in an accumulation of NP in the sludge and a decrease of the NP removal efficiency to 13-86 %. Furthermore, an increase of the NP concentration was observed in the effluent from a reactor receiving only synthetic medium.

Staples et al. (*90*) have examined the aerobic ultimate biodegradation of NP and OP using the OECD 301B modified Sturm test. The test vessels were inoculated with activated sludge from a STP in the U.S.A. The test substances were added in a concentration of 10 mg AP/l and the vessels were incubated at 22 ± 2 °C. The evolution of carbon dioxide, which is a direct measure of the ultimate biodegradation of the test compound, was followed for a period of 35 days. On day 35, the CO_2 formation in the test vessels reached 48 % and 70 % of the theoretically produced CO_2 for NP and OP, respectively. Chemical analyses showed no detectable concentration of either NP or OP in the test vessels at the end of the test period.

The degradation of NP2EO and NP1EO has been investigated under anaerobic condition by Ejlertson et al. (*91*). Anaerobic bottles were amended with 100 % digested sludge at different concentrations of NPnEO (n=1-2). (U-^{14}C)-NPnEO was used to detect any possible decomposition of the aromatic moiety of the NPnEO. NPnEO degraded at 2 mg/l, with nonylphenol as the ultimative degradation products. Both NP and NPnEO interacted with the organic matter, which resulted in sorption to the solid phase.

The possible degradation pathways of APnEO based on the above studies are presented in Figure 11.2.

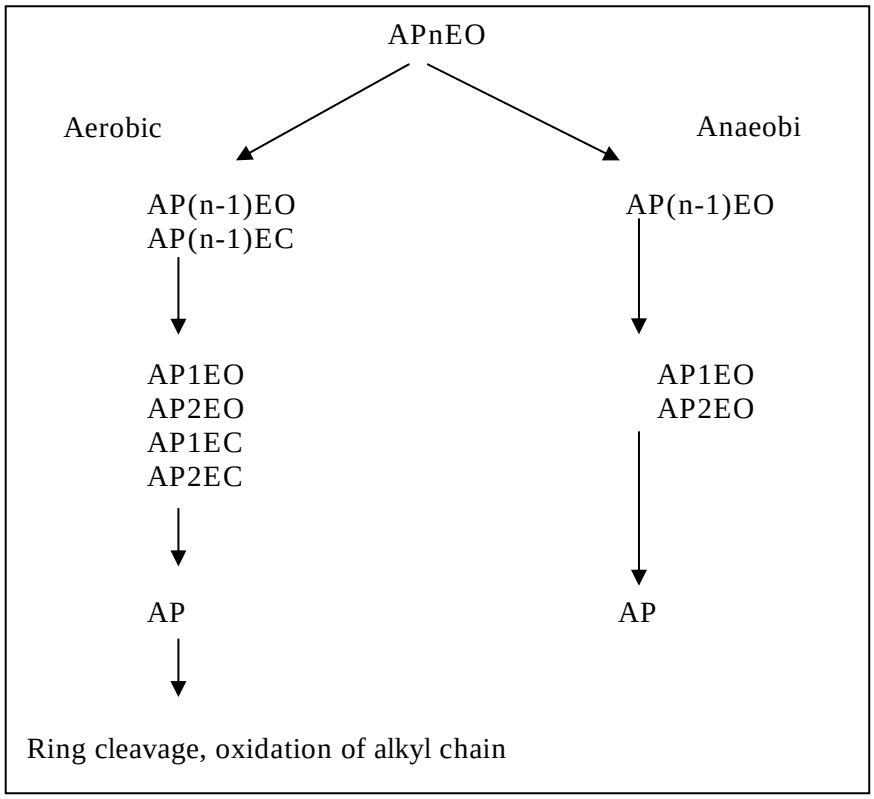

Figure 11.2. Degradation of APnEO (*93*), Renner 1997 in (*238*).

11.2.2 Sorption to sludge particles

The most abundant alkylphenols, nonyl- and octylphenol tend to be associated with sludge and other organic particles due to the high hydrophobicity of these substances. The octanol/water coefficient (log P_{OW}) for NP and OP at 4.48 and 4.12, respectively, exceed the log P_{OW} level for considering a substance bioaccumulative or for having a tendency to sorb to organic matter (log $P_{OW} \geq 3$). The removal of NP and OP from the aquatic phase in a STP may, therefore, not only occur through degradation but also as a result of sorption to the sludge fraction. Measurement of sorption coefficients (K_d) of NPnEO homologues (n = 3-13) onto sewage sludge has showed K_d values ranging from 12,000 to 33,000 L/kg (John et al., 2000 in (*238*)).

11.2.3 Fate in STPs

It is necessary to have a certain knowledge of the hydrophobic nature of the parent compounds APnEO and APnEC to understand the fate of APs in a STP. The hydrophobicity of APnEO depends of the length of the hydrophilic polyethoxylate moiety, i.e. a shortening of the chain results in substances of increasing hydrophobicity ending up with AP as the most hydrophobic of the metabolites. In contrary, a carboxylate group at the end of an ethoxylate chain giving APnEC increases the hydrophilicity.

Investigations of the fate of AP in STPs have focussed on NP and to a certain extend OP. Ahel et al. (*93*) performed an extensive investigation of the

behaviour of nonylphenol polyethoxylates (NPnEO, n=3-20) and their metabolites in eleven full-scale mechanical-biological STPs in Switzerland from 1983 to 1985. The STPs had capacities ranging from 4,000 to 240,000 population equivalents (PE). The typical processes of the STPs were a primary clarifier for mechanical sewage treatment, an aeration tank and a secondary clarifier for biological sewage treatment, and an anaerobic digester for sludge treatment.

The concentration of NPnEO and their metabolites varied significantly in the examined waste water. The total concentrations of nonylphenol compounds (NP-c) for primary and secondary effluents ranged from 1,090 to 2,060 µg/l and from 240 to 760 µg/l, respectively. Thus, the overall elimination efficiency of NP-c from the water phase was relatively low with an average of 59 ± 18 %. The distribution of NPnEO oligomer and metabolites changed during the different treatment processes towards lower oligomers (*n*EO < 8), NPnEC and NP. The most dramatic change in the composition occurred during the activated sludge treatment. A model was proposed for the relative mass flow and the average composition of NPnEO based on the obtained data for the eleven STPs (Figure 11.3.). The
investigation showed that the main part of the remaining NP-c (60 %) was transported to the aquatic recipient via the secondary effluents as un-transformed NPnEO (n: 3-20), NP1EO, NP2EO, NP1EC, NP2EC and NP. The remaining 40 % of the total NP-c load were disposed of to the environment via digested sewage sludge. A significant amount of the NP was discharged from the STPs via digested sludge. This was particularly pronounced for NP, of which 92-96 % were in the anaerobically digested sludge and only 4-8 % in the secondary effluents. The adsorption of NP to the sludge particles in addition to the formation of NP as the end product during anaerobic degradation resulted in high NP concentrations in anaerobically digested sludge (*93;240*).

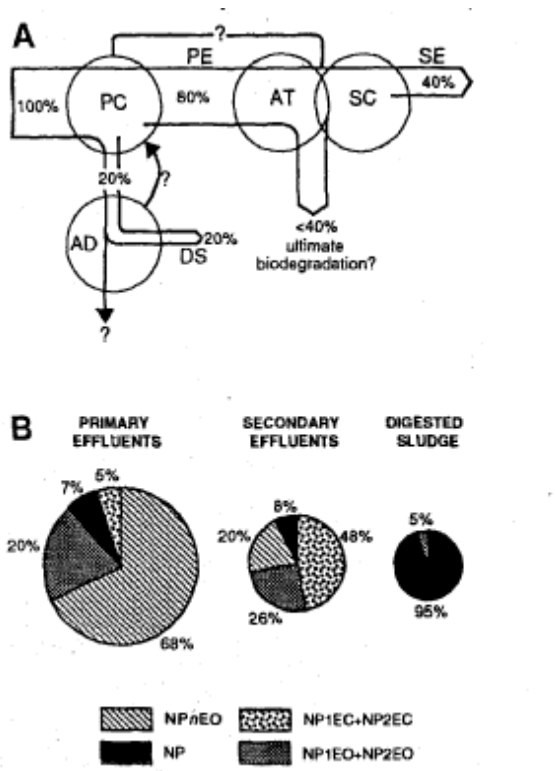

Figure 11.3. Estimated relative mass flow (A) and average composition (B) of nonylphenolic compounds in 11 sewage treatment plants in Switzerland (calculated on molar basis). PC: primary clarifier, PE: primary effluent, AT: aeration tank, SC: secondary clarifier, SE. secondary effluent, AD: anaerobic digestion, DS: digested sludge (*93*)

Carboxylated NP-c with longer chain of ethoxylates, i.e. > 2, have been found at an examination of influent and effluent samples collected monthly for 12 months (1991-1992) from a mechanical-biological STP in Rome. In average the amounts of NPnEC with n > 3 accounted for approx. 30 % of the total content of carboxylated compounds in the effluent. The concentrations of the total amount of NPnEC were in the range of 10 to 145 µg/l. The highest concentration was found in December (145 µg/l) followed by a concentration in July of 58 µg/l (*241*). Quantification of NPnEC oligomers (n: 1-4) in effluent samples from STPs near Green Bay, U.S.A., showed the following average proportion of NPnEC: 7 % of NP1EC, 54 % of NP2EC, 31 % of NP3EC, and 8 % of NP4EC (*242*). The total NPnEC concentration was in a range of 143-272 µg/l. Furthermore, double carboxylated metabolites have been identified in sewage effluent water, in which both the alkyl and ethoxyside chains become carboxylated (CAPnEC). Analysis of a sewage plant effluent in Rome showed a total CAPnEC concentration of 58 µg/l (*243*). Not all of the mentioned degradation products have been included in the existing fate studies of APnEOs and their metabolites. Therefore, many of the studies give an incomplete understanding of the fate of the compounds in STPs.

Surveys of the concentrations of NPnEO and their metabolites in wastewater samples from Canadian STPs have been described by Bennie et al. (*244*) and Lee & Peart (*94*). Bennie et al. (*244*) have presented a snapshot of the

occurrence of some APnEO metabolites in samples from streams from 16 different STPs collected in the period from 1995-1996. 4-tert-octyl phenol (4-t-OP), 4-NP and the mono- and diethoxylates of NP were analysed in samples of influent (9 STPs only), final effluent and sludge. It is not possible to make a mass balance over the fate of NPnEO and their metabolites. However, the study showed that the NP concentrations were higher than the OP concentration in all streams. Furthermore, it was found that the highest concentrations were associated with STPs handling large volumes of textile mill waste effluent. The concentration of NP in influent samples from STPs known to receive textile waste water were high (> 100 µg/l) compared to the range in the influent of the other plants of 0.7-62 µg/l.

The study of Lee & Peart (*94*) includes analytical data of NPnEO (n: 0-17) as well as NPnEC and OPnEC (n: 1-2) (OPnEO coeluted with NPnEO in the analysis of this study). Samples were collected monthly from influent (untreated water), primary effluent and final effluent for 12 months (1997-1998) of one Canadian STP. The STP received water from a municipality with a population of approx. 140,000 and from local industries. The treatment processes of the waste water were as follows: primary sedimentation, aeration with activated sludge as secondary treatment and secondary sedimentation before discharging to the environment. Chlorinating of the final effluent was used from May to October. NP and OP were found in all type of samples, i.e. influent, primary effluent and final effluent. The OP concentration was lower than the concentrations of NP in all samples. The influent generally had the highest NP concentration varying from 1.81 to 22.69 µg/l. The NP concentrations in the primary effluent and the final effluent were ranging from 1.59 to 10.92 µg/l and from 0.56 to 2.12 µg/l, respectively.

There was no obvious seasonal or temperature dependence of the concentrations in the sample. The concentrations (in nmol/l) of the total alkylphenolics in the sewage samples are shown in Appendix A, Table A.2. Summarising the results showed the same picture of the fate of the compounds throughout the STP treatment process as found by Ahel et al. (*93*). The primary sedimentation did not significantly alter the distribution between the different groups of compounds (APnEO, APnEC, AP). However, APnEC was the most abundant group of compounds (NpnEC, 64 % and OpnEC, 13 %) after the activated sludge treatment as measured in the final effluent. The elimination rate based on the molarities of the nonylphenolic compounds showed elimination efficiencies ranging from 20 % to 76 % over the year. The highest elimination was seen in April and October and the lowest in September and December. The average reduction of 53 % was similar to the elimination of 59 % obtained by Ahel et al. (*93*).

The occurrence and fate of NPnEO were examined in two Danish STPs during 1998-1999. One of them Herning STP was loaded with high amounts of industrial sewage (50 %) while the other plant located in Hilleroed mainly received sewage from household (95 %). The investigation of these STPs primarily focussed on the occurrence and fate of the nonylphenolic compounds in the following processes: primary sedimentation, anaerobic digestion of primary and secondary sludge and aerobic treatment of the waste water (*245*). The analytical programme included analysis of NPnEO (1-18) and NP but not of carboxylates. The sum of the NP-c concentrations in samples taken from the influent and primary effluent was in the range of <20-64 µg/l and 37-51 µg/l, respectively. The primary effluent was only examined

at Herning STP in the spring of 1999. The total NP-c concentration in sludge after dewatering, which was the final step in the sludge treatment, was 41-220 µg/kg dry weight. The concentrations in the analysed streams were highest in the STP heavily loaded with industrial sewage (Appendix A, Table A.2). Evaluation of the distribution of the analysed NP-c when passing through the treatment processes within the STPs showed that the amount of NP increased from the influent to the dewatered sludge while the amount of NPnEOs (n = 1-18) decreased. The results indicated that the degradation of NPnEO stops with NP in the anaerobic digester.

Fujita et al. (*246*) studied the occurrence of NPnEOs, their metabolites including carboxylates and halogenated derivates in fourty full scale STPs in Japan from 1995 to 1996. Almost all the STPs consisted of primary sedimentation, aeration tanks with activated sludge, secondary sedimentation and disinfection with chlorine or ozone. The primary effluents (after primary sedimentation) were dominated of NPnEO (n=4-8). Nonylphenolethoxylates and carboxylates with 1-3 ethoxylates were also present but they accounted for less than 5 % (mol/mol) of the total NP-c of 10.3-1,972 µg/l. NP and halogenated compounds were not found in the primary effluent. The summary of analysis of samples taken from primary effluents, secondary effluents (SE) and final effluents (after disinfection) is shown in Appendix A. Halogenated NP-c were found in concentrations of up to 52.4 µg/l in the final effluent. The average concentration of NP-c in the secondary and the final effluent were 95.4 and 90.0 µg/l, respectively. NP was seen in both the secondary and final effluent in minor concentrations of up to 3.9 µg/l. The average removal of the NP-c from the water phase in the STPs were approx. 60 % and 70 % after biological (SE) and full treatment, respectively.

Several others have examined influent, effluent and in some cases also intermediate streams in STPs. Körner et al. (*247*) examined the municipal sewage plant in Steinhaule in Germany processing sewage from 200,000 inhabitants. The plant had a total capacity of 350,000 people equivalents (PE) and was equipped with primary sedimentation, activated sludge treatment, biological nitrate removal, biological phosphate removal and final settlement tanks as the main cleaning steps. Influent and effluent samples from four low and eight high technology STPs in the area of Århus, Denmark, were studied by Boutrup & Plesner (*98*) in 1998-2001. Nasu et al. (*89*) studied 27 Japanese plants in a period from 1998 to 1999. Planas et al. (*248*) studied the degradation of NPnEOs in a treatment plant near Barcelone in Spain receiving industrial and domestic sewage from a city with 134,000 PE. Samples were collected from four sampling points during the treatment process. Finally, Sheahan et al. (*249*) have studied a STP in West Yorkshire, the U.K. The objective of this study was to determine the concentration of nonylphenolic compounds from different trade sources entering the STP and to evaluate the contribution of these compounds to the estrogenic activity of the final effluent. Common to all these studies is that the analyses of NP-c only covered some of the metabolites. There was no analysis of carboxylates NP-c and some of the studies only included NP, NP1EO and NP2EO. Therefore, the data give no additional information to the fate of NP-c and their metabolites in STPs.

11.3 Bisphenol A

11.3.1 Biodegradation in sludge of STPs

Bisphenol A appears to be considered readily biodegradable, possibly after a short period of adaptation as stated in Section 3.3.3.1.

Examination of the biodegradability using a modified SCAS procedure (semi-continuously activated sludge) and microorganisms obtained from a municipal STP showed a removal of bisphenol A of 85-96 % after 24-30 days (Turner & Watkinson 1986 in (*221*)). The initial concentration of bisphenol A was 20 mg/l and the removal was followed by measurement of % dissolved organic carbon (DOC) removal and UV adsorption spectroscopy. A lag phase of 13 to 17 days was observed before the initiation of the degradation. Another biodegradation study of an activated sludge batch test study using sludge from an industrial STP resulted in a removal of bisphenol A of 72 % chemical oxygen demand (COD) and 57 % total organic carbon (TOC) after 24 hours. The concentration of the activated sludge was 2-3 g SS/l (SS: suspended solid) and the initial test concentration was 58 mg/l of bisphenol A (*250*).

These results of biodegradation studies using microorganisms from STPs show that bisphenol A should be degradable in STPs. Furthermore, the studies indicate that the degradation rate will increase after a period of adaptation.

Degradation of bisphenol A under anaerobic or anoxic conditions is much more unlikely to occur as explained in Section 5.3.3.2.

11.3.2 Sorption to sludge particles

The reported log P_{ow} of bisphenol A of 3.4 indicates that an amount of the compound in a STP may be removed from the water phase by sorption to sludge particles. An estimation of the fate in STPs according to the principles in the EU's Technical Guidance Document (*251*) showed the following distribution of bisphenol A: 12 % to water, 6.2 % to sludge, 81.9 % degraded and a negligible fraction to air (*221*).

11.3.3 Fate in STPs

The investigation by Lee & Peart (*96*) of samples from influent, effluent, and sludge taken from eight Canadian STPs in 1999 gives an impression of the fate of bisphenol A in STPs. The investigations were performed in connection with the development of analytical methods for bisphenol A. The concentrations in the influent and effluent samples were in the range of 193 to 2,440 ng/l and 35 to 223 ng/l, respectively. The removal efficiency from the water phase was 47-96 %. Analysis of digested sludge showed concentrations between 316 and 12,500 ng/g dry weight. For two of the STPs, data are available on raw sludge and digested sludge. In both cases, the concentration in the raw sludge is approx. 70 % lower than the concentration in the digested sludge. Bisphenol A is not expected to be degradable under anaerobic conditions. Supposing that the digestion process is anaerobic, the observed increase of the concentration could be due to a lower water content in the digested than in the raw sludge.

In Germany, Körner et al. (*247*) found bisphenol A in two influent samples of 540 and 3,010 ng/l. The concentrations in the corresponding effluent samples were 162 and 258 ng/l giving removal efficiencies of 70 % and 91 %, respectively. Examination of four low technological sewage treatment plants in Denmark showed influent concentrations of <100-1,300 ng/l and effluent concentrations between 50 and 1,800 ng/l. The percentage removal of bisphenol A was between ~ 0 % and 96 % (*98*). The lowest removal was found in a plant with only mechanical treatment. Recently, Fromme et al. (*252*) have reported the results from an investigation of sludge and effluent samples collected from 39 German STPs. The concentration in the sludge samples was 4-1,363 ng/g dry weight, which is comparable with some of the concentrations observed by Lee & Peart (*96*).

12 Influence of the type of treatment plants on the removal efficiency of estrogens and xenoestrogens

The fate of estrogens, bisphenol A, alkylphenols and the parent compounds of alkylphenols in STPs was considered in Chapter 11. The fate of the compounds in STPs is mainly correlated with the intrinsic properties of the compounds, i.e. physico-chemical properties and degradability. There is, however, no doubt that the type of treatment plant and the operation conditions are important for the overall removal of the compounds in STPs and thus the final concentrations in effluent and sludge.

An evaluation of the influence of the type of treatment process is given in this chapter. The evaluation is based on data and information on STPs collected from a number of studies. Details about the STPs are given in Appendix A, Table A.1. The concentrations of the compounds from each of the STPs are listed in the Tables A.2, A.3 and A.4.

There are several difficulties in comparing the different studies. As already mentioned in the previous chapters, different analytical methods and sampling strategies have been used in the different studies. Treatment conditions of the STPs studied are often not completely described. E.g. hydraulic retention time (HRT), sludge retention time (SRT), temperature, denitrification, nitrification and phosphate elimination will all have an important bearing on the plant efficiency (*95*).

12.1 Estrogens

The concentration ranges of the estrogens in effluent of STPs are approximately at the same levels in the different countries (Table 5.1., and Appendix A, Table A.2). The concentrations varied from the lowest detection limits up to approx. 220 ng of estrone/l. This high concentration was found in a British STP in Chelmsford in 1997-98. The analysed samples from Chelmsford were 5 days composite samples taken at noon every day, i.e. grab samples. The high concentration may therefore represent a single high peak of estrogens in the effluent.

Apparently, there is a tendency to higher concentrations of estrogens in effluents from the U.K. compared to the other European countries. However, the available data do not allow any final conclusions regarding potential differences between countries.

Comparing data within single studies, in which the sampling techniques and analytical methods are identical, should give the best basis of an evaluation of the importance of the different treatment processes. Desbrow et al. (*253*) studied the concentrations of estrogens in the effluent of seven British STPs with a range of treatment processes. The samples were taken throughout a 24-h cycle. The results of the investigation indicate that the effluent concentration is reduced when the treatment is increased with e.g. tertiary

lagoons as in Rye Meads STP. However, the analyses were only performed on sample fractions with estrogenic activities and do not necessarily include the total amount of estrogens.

STPs with only primary and secondary treatment as well as plants with primary, secondary, tertiary and advanced treatment (e.g. microfiltration followed by reverse osmosis) have been examined in California (*97*). All the plants were equipped with a final disinfection step. Estradiol and ethinylestradiol were the only estrogens analysed in the study. The investigation indicated that the removal efficiency of these compounds increases with improving of the primary and secondary treatment with a tertiary treatment step. Advanced treatment with reverse osmosis resulted in a very low concentration of <0.4 ng/l in the effluent before the disinfection.

Danish investigations performed at low and high technology STPs in Århus during 1998-2001 show that the lowest effluent concentrations of estrogens (max. 9.2 ng/l) were obtained in the high technology plants. The low technology plants had concentrations of estrogens of up to 140 ng/l (sum of estrone, estradiol and ethinylestradiol). These low technological plants are placed in the "open land" treating household waste water from areas with scattered houses (*99*). The details of the different STPs in this investigation are included in Appendix A, Table A.1.

An investigation of five British STPs with different types of treatment by Kirk et al. (*254*) indicates that the major reduction of estrogenic activity occurs during the secondary treatment, i.e. the biological process, presumably mainly due to biological degradation. There were, however, two exceptions showing considerable reductions after both primary and secondary treatment. The hydraulic retention times in these plants were longer (13 h) and maybe also in the primary treatment and this may be the reason for the increased removal. Generally, the plants with the longest hydraulic retention time (HRT) (13-13.5 h) showed the greatest removal of estrogenic activity. Tertiary treatment (ammonia removal in a Biostyr plant and ultraviolet treatment) was seen to further remove estrogens. Kirk et al. (*254*) conclude that the more efficient STPs are capable of removing most of the activity (>70 %) but less-modern plants, with no tertiary treatment or less efficient processes than the currently available, are less efficient and removal rates are lower. It should be noticed that Kirk et al. (*254*) measured the estrogenic activity using yeast-based assay and not specific chemical analyses. The study, therefore, illustrates the influences of the type of plant regarding the overall removal of estrogens and xenoestrogens together.

The hydraulic retention time in a STP plays an important role for the biodegradability of a compound. Long retention in the biological treatment step increases the time of degradation. Only few of the investigators have reported the HRT of the plants. The reported HRTs are in the range of 11 to 26 hours. Another important process parameter is the sludge retention time (SRT). A long SRT of e.g. 25 days compared to a short retention time of e.g. 5 days may possibly increase the possibility of establishing and maintaining a microbial flora capable of degrading the estrogens. Increasing the SRT could also increase the removal of e.g. estradiol and ethinylestradiol from the water phase by sorption to the sludge particles. The effect of the HRT and SRT in STPs cannot be evaluated on the basis of the available data in literature. However, an investigation of influent and effluent samples from three Dutch STPs based on the activated sludge system showed that the highest removal

efficiency of estrone and estradiol was obtained in the two plants with the highest HRTs (18 and 26 h) and SRTs (11 and 20 d) (*88*)

The biodegradation rate of a compound is a function of the temperature and the degradation rate may be reduced considerably at low temperatures. Studies by Ternes et al. (*86*) and Rodgers-Gray et al. (*255*) indicate that temperature variations in STPs affect the concentration of the estrogens in the final effluent. Ternes et al. (*86*) observed lower absolute removal rates in a German plant than in a plant in Brazil and concluded that it might be due to the low temperature of –2 °C in Germany at the sampling time compared to above 20 °C in Brazil. A very high concentration of estrone at 220 ng/l has been found in the effluent of Chemsford STP as mentioned above. This concentration was found during the winter season, when the temperature was 12.3 ± 0.4 °C. The concentrations were generally higher during this period compared to a period with higher temperatures of 17.2 ± 0.7 °C. The observed differences could be a result of the variations in the temperature. However, Johnson et al. (*88*) did not find a correlation between the temperature and estradiol removal obtained in Dutch STPs.

12.2 Alkylphenols

One of the most extensive investigations of the fate of alkylphenols and their parent compounds in STPs was performed by Ahel et al. (*93*). The study included analyses of NP, NPnEO (n = 1-18) and NPnEC (n = 1-2) as stated in Chapter 2. However, only few details on the operation of the treatment plants were given in the study. The plants were of the same type, i.e. consisting of primary sedimentation, activated sludge, final sedimentation and anaerobic digestion. The overall removal of the NP-c was 26-79 % based on the molarity in the effluents from primary and final sedimentation, and the total concentrations of NP-c for primary and secondary effluents ranged from 1,090 to 2,060 µg/l and from 240 to 760 µg/l, respectively. A detailed evaluation of the data showed that the highest elimination rates were achieved in the STPs characterised by low-sludge loading rates and nitrifying conditions. The percentage of NP-c remaining in secondary effluents correlated (r= 0.9035) with the actual STP loads, expressed as the percentage of their design capacity. Furthermore, the data on the removal of the hydrophobic compounds (NP, NP1EO, NP2EO) in periods with varying temperature indicated a temperature dependence of the removal (*93*).

A study of sewage samples from a Canadian STP from March 1997 to February 1998 confirmed the results obtained by Ahel et al. (*93*) (*94*). The Canadian STP was of the same type as the STPs investigated by Ahel et al. (*93*) and showed a similar mean elimination rate. The elimination varied widely as mentioned in Chapter 2 but the removal of APEO and their metabolites in the STP did not seem to be ambient temperature dependent.

Introduction of a disinfection step using chlorination may result in the formation of halogenated (chlorinated or brominated) NPnEOs and NPnECs as seen in an investigation of forty STPs in Japan (*246*). The STPs studied by Fujita et al. (*246*) consisted of primary clarifiers, aeration tanks, secondary clarifiers and disinfection processes with a few exceptions without disinfection processes. Halogenated compounds were found in 25 STPs.

Bennie et al. (*244*) investigated 16 STPs in Canada with different treatment systems. Although only NP, NP1EO and NP2EO were analysed, some

tendencies were obvious. Plants with only primary treatment tended to be less efficient. The tertiary treatment systems at Cambrige-Galt and Guelph STPs were among the most efficient as regards removal of NP and OP from the water phase. The sludge was treated in anaerobic digesters at nine of the STPs. There was no biological treatment of the sludge at the remaining seven plants. NP, NP1EO and NP2EO were found in concentrations of up to 909 mg/kg dry weight in the anaerobically digested sludge.

It is possible to reduce the concentration of AP, AP1EO and AP2EO in anaerobically digested sludge by introducing a post-aeration step. The effect of the post-aeration has been investigated in lab-scale (batch test) as well as in the continuous full-scale process at Usserød STP in Denmark. It was demonstrated that it is possible to reduce the content of e.g. APnEO (n = 0-2) with 75-95 %. Furthermore, it was demonstrated that the dewaterability of the post-aerated sludge was nearly as efficient as for digested sludge.

The Danish investigations of STPs in Århus during 1998-2001 also included analyses of AP and APnEO (n = 1-15) (*98;99*). The tendency in the results was the same as obtained for the estrogens. The highest effluent concentrations of NPnEO (n = 0-2) of 1.83-7.62 µg/l were found in the plant with the lowest technology, i.e. Tåstrup STP consisting of only a mechanical treatment system. However, concentrations were seen at the same level in one of the high technology plants Søholt STP. Analysis of influent samples from this STP showed correspondingly high concentrations. Identification of the sources of NPnEO in the catchment area showed that 52 % of the NPnEO (n = 0-15) in the influent was discharged from a single industry. The concentrations of NPnEO (n = 0-2) in the effluent from the other high technology plants were in the range of approx. 0.2 to 1.2 µg/l. The investigations did not include analysis of carboxylated alkylphenol ethoxylates and data from these studies do not allow further assessment of the influence of the type of STP. However, the investigations confirm the finding of other investigators that the removal efficiency of the compounds to a certain extent depends on the level of applied technology at the STPs.

12.3 Bisphenol A

The highest of the reported effluent concentrations of bisphenol A was found in the Danish Randers STP at 4,000 ng/l (*98*). The plant is upgraded to nutrient removal and 26 % of the influent water is coming from industries. The bisphenol A concentrations in two influent samples of 700 and 3,000 ng/l are not remarkably high compared to the influent concentrations obtained in samples from other STPs. However, the analytical results for Randers STP are probably not from parallel sampled influent and effluent samples. It is obvious that the lowest of the reported removal efficiencies was found at the low technology plant, Tåstrup in Denmark with only mechanical treatment.

The available data for bisphenol A do not allow any further assessment of the influence of the type of STP on the removal of bisphenol A.

13 Advanced treatment processes

Conventional sewage treatment is not an effective barrier to trace contaminants with e.g. high estrogenic potency. Removal rates published in the literature vary greatly depending on e.g. local treatment facilities, discharges from the industry and the nature of the contaminant as discussed in the previous chapters (*102*). There has, therefore, been an increasing focus on the use of more advanced treatment processes with the objective to remove the trace contaminants and among them the estrogenic compounds.

The effectiveness of electrochemical methods in purification of synthetic waste waters containing bisphenol A has been tested in a study by Boscoletto et al. (*256*). The concentration of bisphenol A ranged from 20 to 2,000 mg/l and are thus considerably higher than the concentrations found in the effluent of STPs of 4.8- 4,000 ng/l. Electrochemical tests were conducted galvanostatically. A titanium mesh was used as a cathode and a platinium mesh or a titanium-supported lead dioxide film as anode. The treatment was performed in 2.8 % NaCl at pH >10.6 and the effect of different times of electrolysis was tested. Complete destruction of bisphenyl A was detected after 24 h. The final products consisted of simple short chain aliphatic acids. However, formation of chlorinated aromatic intermediates was observed during the treatment process. Boscoletto et al. (*256*) concluded that further investigation was necessary a.o. to find the optimal electrolysis conditions leading to a reaction path free of dangerous products.

Enzymatic treatment represents one method by which selective removal of contaminating compounds may be obtained and has been widely studied (Aitjen 1993 in (*257*)). Caza et al. (*257*) performed a study with the objective to optimize the reaction parameters, in unbuffered tap water, to achieve at least 95 % removal of several different aromatic compounds including bisphenol A by using soybean peroxidase (SBP). The reaction parameters, which were optimized, were pH, SBP dose both in the presence and absence of polyethylene glycol (PEG), hydrogen peroxidase to substrate ratio (H_2O_2/substrate) and PEG dose. The investigation demonstrated that it was possible to optimise the treatment parameters to achieve the wanted removal efficiencies. However, despite the suitability of enzymatic methods in the treatment of clear water with solutions of e.g. bisphenol A, the potential for STPs effluents is questionable.

Essex and Suffolk Water in the U.K. investigated estrogenic aspects of its treated wastewater-recycling scheme. The waste water was discharged directly into the Hanningsfield reservoir for a period of one year after UV disinfection as a temporary recycling scheme. It was the intention that a permanent recycling scheme with output from a new STP would be discharged into the River Chelmer from which water is abstracted. A research programme was carried out which included investigations of the effect of advance treatment processes on the removal of AP, APnEO, estradiol, estrone and ethinylestradiol. A pilot plant treatment of spiked sewage effluent showed that the plant removed substantial amounts of the compounds. The pilot plant included pre-ozone (1 mg/l for 4 min), ferric sulphate clarifier (8 mg/l, pH 6.2-6.5), sand filter, post-ozone followed by granular activated carbon

(GAC). The pilot plant flow was 2,000 L/h. Monitoring for estrogens in the inlet and outlet of the UV disinfection plant before discharging the water indicated that UV light could reduce the estrogenicity. Laboratory experiments with estrone, estradiol, and ethinylestradiol standards showed removal in the range of 4-24 % after UV-treatment. The treatment parameters were 145 m Ws/cm^2 and 20 s retention time (*101*). The initial concentration of each steroid was approx. 25 ng/l, which is within the range of concentrations found in effluents of STPs. Furthermore, application of activated carbon (50 mg/l) resulted in a mean removal of estrogens from an operated pilot plant of 94.4 % during a period of eight days.

Shishida et al. (*100*) compared the capability of sand filtration, microfiltration (MF), reverse osmosis (RO) and ozone/hydrogen peroxide treatment (AOP) to reduce the estrogenicity and genotoxicity of the secondary effluent from a municipal STP with a pilot-scale reactor. A stream of the effluent water was let to the pilot plant. The water was pumped into the following sequence of treatment steps: sand filter unit, and an AOP reactor and a membrane treatment unit in parallel. The membrane treatment unit consisted of two subunits: a MF unit followed by a RO unit. Samples were collected at the outlets of secondary clarification, sand filtration, AOP, MF and RO units. The reduction of the toxicity in the waste water was evaluated by testing with different bioassays. The results of the bioassays showed that both AOP and RO treatments effectively reduced genotoxicity, cytotoxicity and estrogenicity of the secondary effluent from the full scale STP. No significant differences were observed among the secondary effluent, sand filtration and MF effluents with respect to those toxicities. Shishida et al. (*100*) concluded that the installation of sand filtration and MF modules in a STP is not sufficient for the reduction of those toxicities.

An Australian three-year project: "Optimised Use of Membrane Hybrid Processes for Water Recycling" (ARC SPIRT Project) was initiated in 2000 with the Queensland Government as the industry partner. Endocrine disrupter removal is the core issue of this project. The aims of the project are to investigate trace contaminant removal by hybrid membrane processes from waste waters (*102*).

A number of processes have been investigated regarding their potential for removal of endocrine disrupters. Those processes are ferric chloride coagulation, powdered activated carbon, magnetic ion exchange combined with microfiltration (MF) or ultrafiltration (UF) as well as nanofiltration (NF) and reverse osmosis (RO). The key findings have been a negligible removal (<10 %) of estrone with ferric chloride coagulation and very high removal (>90 %) with powdered activated carbon. Magnetic ion exchange varied from 40 to 70 % removal dependent on solution chemistry and dissociation of the hormone. Nanofiltration showed an initial retention of 70-95 % but, for most membranes, this retention dropped significantly after an initial filtration period. For some reverse osmosis membranes, retention was similar to nanofiltration, but others showed a very high and stable retention of the compounds. Microfiltration also showed initial almost complete retention followed by a drop as expected. The presence of matrix compounds from water and waste waters affected retention for some membranes. The results showed adsorption of polar contaminants to materials used in the treatments. This might be a significant risk in water recycling, in which contaminants accumulate to comparably high quantities and may be released during

treatment. This requires further investigations. The obtained results are currently being confirmed on larger scale systems (*102*).

A three-year ongoing EU study (POSEIDON) is working with the development of possible clarification techniques for increasing the removal of endocrine disrupters. Ried & Mielcke (*103*) have studied the application of ozone and UV. Pilot tests were carried out at the municipal STP in Braunschweig in Germany. Braunschweig STP is designed for 385,000 PE and comprises different treatment steps with mechanical pre-treatment and biological treatment for carbon and nutrient removal. Under normal conditions, the flow rate is approx. 60,000 m^3/d. Braunschweig STP is the potential end user of the EU project POSEIDON. The tested ozone/UV pilot plant installed at the STP comprises an ozone generator (100 g/h), 2 diffuser/bubble columns followed by an UV-reactor. The initial ozone treatment improves the conditions for the following UV-treatment by decreasing absorption coefficients, which eventually improves the UV transmittance from 59 % to 84 %. The preliminary results show a positive treatment effect as regards endocrine disrupters. The operating costs of the production of ozone were estimated to be between 0.90 and 1.60 EURO per kg ozone depending on the energy prices and the system capacity.

14 Possible non-sewage effluent related sources of estrogens to the aquatic environment

The possibility that other sources to estrogens in the aquatic environment exist besides direct release with sewage effluent also has to be considered when evaluating the exposure risk for the aquatic fauna towards exposure to estrogens.

A source which has been considered is the manure from cattle, pigs, poultry and other domestic animals (*258-261*). Runoff from spreading of manure on fields for fertilisation might take place and has been demonstrated for poultry manure (*259-261*).

The concentration of estrogens in manure from domestic animals has been estimated in a report by the Dutch Association of River Waterworks (*258*). Estrogen emission from pregnant cows in faeces and urine as both conjugated and unconjugated estrogens has been estimated to 37.3 mg/day based on an average concentration during gestation of 54 µg/kg manure (in (*258*)). In non-pregnant cows the daily excretion was assessed to 1.1 mg/day or 30 µg/kg. A dutch study has reported concentration of estrogens (DW) in manure from cattle to 46 – 50 µg/kg 17ß-estradiol, 28 – 72 µg/kg estrone and <1 µg/kg ethinylestradiol (*6*). The estrogen emission from breeding and non-breeding sows as manure and urine has been estimated to 6.8 mg/day (1.13 mg/kg manure) and 31.7 µg/day, respectively. From chicken the emission of estrogens by urine is negligible compared to emission by manure in which a total content between 14 µg/kg dry weight for male chicks and 533 µg/kg for laying hens (*262*).

One experiment with application of 5000 kg/ha of chicken manure to fields yielded runoff concentrations of 3.5 µg E2/l and 1413 mg/ha after simulated rainfall (50 mm/h) (*261*). The runoff was generated immediately after litter application and designed to represent worst case scenario. Another study by Nichols et al. has demonstrated first runoff concentrations of 1.28 µg/l and 198.8 mg/ha after application of 7050 kg/ha (*260*). Second runoff concentrations were 66 and 69 % less than that of first runoff.

Part of the estrogens applied with manure will bind to the soil. Experiments in which heavy soil from a field was irrigated for several months with sewage water indicated that 56 ± 2 % of the estradiol and 59 ± 2 % of estrone was strongly bound by the soil and only extractable by organic solvents. Testosterone, on the other hand, was readily washed out by aqueous solutions (*262*).

In general, however, not much literature exists on the subject of manure as a potential risk source for estrogens to the aquatic environment and it is yet difficult to estimate the importance of this as a possible contributor to stream and river contents of estrogens.

Sludge from sewage treatment plants are besides manure also used to fertilise fields in Denmark. As mentioned in chapter 5 estrogens and xenoestrogens have a high log K_{ow} and therefore a strong absorbance to organic material. In the sewage treatment process a proportion of the estrogens will therefore accumulate in sludge (see chapter 11) and
estrogens have been demonstrated to persist during sludge digestion (*263*). In activated and digested sewage sludge from a German study, estrone and 17ß-estradiol have been detected at concentrations up to 37 ng/g and 49 ng/g, respectively. Ethinylestradiol was detected at concentrations up to 17 ng/g (*263*). Nonylphenol has been detected at concentrations of 137 ± 7.7 and 470 ± 22 µg/g in measurements of sludge from two Canadian STPs (*264*), 172 µg/g in sludge fra a Spanish STP (*265*) and < 0.125 – 4.59 ± 0.11 µg/g in sludge from two German STPs (*266*). In Denmark in 1995 alkylphenols were reported in the range of 0.3 – 67 µg/g with a median concentration of 8 µg/g. The cut off value for using sludge as fertiliser on fields was in 2000 set to 10 µg/g (*267*). Octylphenol has been detected at concentrations of 9.2 ± 0.4 and 12.1 ± 0.5 µg/g and 7.5 µg/g in sludge from two Canadian and one Spanish STP, respectively. Bisphenol A seems to be present in even lower concentrations and has been reported to < 0.125 – 0.078 – 0.09 µg/g in sludge from two German STPs.

Again, however, little information exist on the runoff of estrogenic compounds from the sludge once deposited on the field. An experiment in which sewage sludge was applied to sandy soil in lysimeters, few leachate samples induced growth of human breast cancer cells (MCF-7) cells in the E-screen. The highest estrogenic activity in leachate samples, detected as estrogen equivalents, was 2.94 ng/l (*268*). The measured concentrations of various estrogenic compounds in the sewage sludge were: nonylphenol, 1.51 mg/g; bisphenol A, 0.38 µg/g; 17α-estradiol, < 2 ng/g and 17 ß-estradiol, 3.5 ng/g. Most leachate and soil extract samples gave no response in the E-screen.

Conclusively, too little is known about runoff of estrogenic compounds from both manure and sludge, used as fertiliser on fields and it is not possible yet to assess whether these are considerable sources of estrogenic activity to the surface waters.

15 Conclusions (English)

Feminisation of male fish in freshwater and marine environments

- Feminisation of male fish has now been detected in a number of countries world-wide. These kinds of hormone disruptions have been seen in a range of both freshwater and marine species of fish though most frequently among freshwater species.

- The feminisations are believed to be caused by release of natural and synthetic estrogens and estrogenic compounds being released to the aquatic environment via sewage effluent.

- Signs of feminisation in male fish are generally a synthesis of the yolk protein vitellogenin, an estrogen marker, and intersex, an abnormal type of hermaphoditism in which males develop egg cells in the testes.

- Worst cases of feminisation in regard to both occurrence and degree of the disruption have been seen in England while a lower extent has been found in other countries including Denmark.

- Care must be taken in using results from short-term exposure studies as ultimate estimates of risk for wild populations of fish which live their entire life in sewage effluent receiving waters.

The estrogenic components of sewage effluent

- A combination of cell based *in vitro* assays and chemical analyses of sewage water has verified and quantified the estrogenicity of sewage effluent from numerous countries. These have also demonstrated that the natural estrogens, 17ß-estradiol and estrone, and the synthetic estrogen, ethinylestradiol used in contraceptives are likely candidates for some observed disturbances in fish from sewage effluent receiving rivers. In single cases, the estrogenic chemicals, alkylphenols, have also been suggested as possible causative agents.

Sewage effluent and surface water concentrations of estrogens

- Concentrations of the three natural estrogens, 17ß-estradiol, estrone and estriol have internationally been detected in sewage effluent at concentrations of < 0.1 – 88 ng/l (typical 1 – 10 ng/l), < 0.1 – 220 ng/l (typical 5 – 20) and < 0.1 – 42 ng/l, respectively. Ethinylestradiol has been found at concentrations of < 0.053 – 62 ng/l (typically below 1 or seldom above 10 ng/l).

- Concentrations in surface waters have been found in the ranges 0.05 – 15.5 ng estradiol/l, < 0.1 – 17 ng estrone/l, < 0.1 – 3.4 ng estriol/l and < 0.053 – 30.8 ng ethinylestradiol/l with typical concentrations of less than 5 ng/l for estradiol and estrone and less than 1 ng/l for ethinylestradiol.

Fate of estrogens in the aquatic environment

- Ethinylestradiol is more persistent than the natural estrogens both in water and sediment. Average half-lives of 2.8 and 3.0 days in water has

been calculated. Ethinylestradiol has been demonstrated to have a ten times as long half-life compared to estradiol. In anaerobic sediment 17ß-estradiol is rapidly converted to estrone, but both estrone and ethinylestradiol show very low degradability in the sediment and might accumulate.

Occurrence and fate of alkylphenols and bisphenol A in sewage effluent and surface water

- Some of the more potent estrogenic compounds which might be released with sewage effluent are the alkylphenols, nonylphenol and octylphenol, and bisphenol A. Nonylphenol and octylphenol have in sewage effluent generally been detected in concentrations below 10 µg/l and below 1 µg/l in surface water, though, few example of concentrations above 300 µg/l in sewage effluent and 600 µg/l in surface water have been seen. Bisphenol A is seldom detected above 1 µg/l in either sewage effluent or surface water. Both alkylphenols and bisphenol A have great potential for accumulating in the sediment.

Lowest effect concentrations for feminisation by estrogens, alkylphenols and bisphenol A

- Laboratory experiments have for 17ß-estradiol found a lowest effect concentration for induction of vitellogenin on 5 ng/l and for induction of intersex on 10 ng/l. A range of other effects have been seen at concentrations between 10 and 50 ng/l.

- Estrone has an equal or slightly lower estrogenic potency compared to estradiol, and lowest effect concentration for vitellogenin and intersex induction in male by estrone is 30 and 10 ng/l, respectively. There is little knowledge on the potency of estriol but *in vivo* it appears to be 100 times less potent than estradiol.

- Ethinylestradiol is more potent than the natural estrogens in regard to inducing feminising effects. Vitellogenin production and intersex in males have been induced by 0,1 ng/l and changed sex ratio by 0,6 ng/l. A range of other testis effects have been seen at concentrations below 10 ng/l.

- Nonylphenol, octylphenol and bisphenol A have lower estrogenicity compared to the natural and synthetic estrogens. Induction of vitellogenin has been made by 5 µg/l nonylphenol or octylphenol, and intersex, changed ratio and other effects at nonylphenol concentrations between 30 and 100 µg/l. Male reproductive disorders have been seen with 2 µg/l octylphenol. Bisphenol A has exerted effects at concentrations between 10 and 40 µg/l.

Relationsship between lowest effect concentrations of estrogens/estrogenic compounds and their presence in the environment

- Comparing sewage effluent and surface water concentrations of the estrogens, alkylphenols and bisphenol A with the lowest effect concentrations for reproductive disruptions by the individual compounds demonstrate that concentrations of estradiol, estrone, ethinylestradiol, nonylphenol and octylphenol in some cases have been high enough to be suspected of causing feminisations in wild fish populations. Based on the present knowledge on environmental occurrence of and the estrogenic potential of estriol it is not possible to estimate the contribution of this estrogen to observed feminisations. Bisphenol A is generally detected at

concentrations below the lowest effect concentrations for inducing reproductive disorders in male fish.

- Which compounds are responsible for observed feminisations of fish in Denmark is still uncertain due to a limited knowledge on water concentrations of estrogens.

- Several aspects have to be taken into account when assessing the possible implications for the reproductive health of fish of estrogenic compounds in the environment. Estrogens and estrogenic chemicals in sewage effluent will act in an additive manner thereby lowing the concentration of a single compounds which is needed to induce effects. Different species exert different sensitivities. The timing of the exposure relative to critical periods in the fish life-cycle is of great importance to the resulting effects, and intermittent exposures to high concentrations of estrogens seems to give unproportionately large effects.

Effect of feminisation or estrogenic exposure on fertility of male and female fish

- Little is known about the importance of the observed signs of feminisation on the fertility and reproductive success of fish. However, observations among intersex roach in England of asynchrony of gamete maturation between males and females due to reduced spermatogenesis as well as reduced sperm volume, density and motility have indicated reduced fertility. The normal development of egg cells in females was also affected. Controlled exposure experiments with the compounds in question have also demonstrated reduced fertilisation success among males and reduced spawning of eggs among females.

- Impact of estrogens and estrogenic compounds on the reproductive capacity might also be indirectly via reduced energy sources to reproduction.

Sources of estrogens to sewage effluent

- The pool of estrogens which enters the municipal sewage system origins form the natural production of estrogens by humans, from hormone and estrogen replacement therapies and the intake of hormone contraceptives containing ethinylestradiol.

- The estrogens are mainly excreted as water-soluble conjungates. Estimation of the excretion of estrogens by humans in Denmark showed that the main part of the estrogens originates from the natural production in humans which contributes to approx. 87 % of the total excretion of estrogens. Excretion from hormone therapy accounts for approx. 12 % while excretion of hormones from contraceptives only accounts for approx. 1 % of the total excreted amount of estrogens.

- The estimates of the total humane excretion of the four estrogens per 24 h were: 36 g of estradiol, 69 g of estrone, 340 g of estriol and 3.2 g of ethinylestradiol.

Sources of alkylphenols and bisphenol A to sewage effluent

- Alkylphenols are mainly used in production of other products as e.g. alkylphenolethoxylates. Alkylphenolethoxylates are relatively easy degraded to alkylphenols and, therefore, important sources of alkylphenols

in sewage systems. No release from production of either alkylphenols and alkylphenolethoxylates in Denmark is expected.

- The nonylphenol family represents approx. 85 % of the alkylphenol market and the remaining 15 % are assumed to be octylphenol.

- The total release of nonylphenolpolyethoxylates to waste water in Denmark was estimated to be between 37 and 996 tonnes per year.

- No production of bisphenol A is expected to take place in Denmark. The release in Denmark is expected to arise solely from processing, use and disposal of bisphenol A containing materials. The release of bisphenol A to waste water in Denmark per year was estimated to 17 kg from PVC processing, 256 kg from PVC use and 492 kg from paper reuse.

Fate of estrogens in sewage treatment works

- Conjungated steroids have been found to contribute to approx. 50 % in influent and approx. 70 % in effluent of German STPs. The total amount of estrogens (estrone, estradiol and ethinylestradiol) was 25.5 ng/l and 9.3 ng/l in the influent and effluent, respectively.

- Based on laboratory experiments it is expected that the conjugates of estradiol are de-conjungated relatively fast and that estradiol is oxidised into estrone which is further eliminated.

- Typical average removal efficiencies of the four estrogens from the water phase of STPs are: 61 ± 38 % of estrone, 87 ± 9 % of estradiol, 96 ± 6 % of estriol and 85 ± 14 % of ethinylestradiol. Analyses of glucuronates were not included in these studies. The removal of the estrogens may occur by degradation as well as sorption to sludge particles.

Fate of alkylphenols and bisphenol A in sewage treatment works

- The resulting products of the degradation of alkylphenolethoxylates under aerobic conditions in STPs are mono- and diethoxylates, the more hydrophobic carboxylates and alkylphenols. The final product alkylphenol seems to be degradable under aerobic conditions. The transformation under anaerobic condition results in production of mono- and diethoxylates and finally alkylphenols. The concentrations of APs in anaerobically digested sludge are often extremely high.

- The average removal efficiencies (water phases) of nonylphenolic compounds of 53 % and 59 % have been found in examination of STPs with analytical programs including nonylphenolethoxylates and their metabolites inclusive mono- and dicarboxylates.

- There are to our knowledges no studies of the fate of alkylphenolethoxylates (APnEOs) within STPs which include all the know metabolites of APnEOs.

- Bisphenol A is expected to be easily degraded under aerobic conditions in STPs but not under anaerobic or anoxic conditions. Furthermore, bisphenol A may be removed from the water phase by sorption to sludge particles. Removal efficiencies from the water phase of 47-96 % have been

observed in Canadian STPs and of ~0-96 % in Danish low technology plants.

Influence of the type of sewage treatment plant on the removal efficiency of the estrogens/estrogenic compounds

- Generally, the removal efficiencies of estrogens and alkylphenolic compounds seem to increase and consequently the effluent concentrations to decrease by increasing upgrading of STPs. E.g. effluent concentrations of < 0.1-0.32 ng estradiol/l have been observed in a California STP after reverse osmosis.

- The use of low technology plants in the so-called "open land" may result in relatively high local concentrations of estrogenic compounds in the aquatic recipient.

- Different operation conditions in STPs as e.g. HRT, SRT, temperature and loading rate will have an important bearing on the plant efficiency and thereby the removal of estrogens and xenoestrogens. However, there is only little information about the operation conditions in the studies of STPs.

- More knowledge concerning the fate of estrogenic compounds within STPs is needed. High quality studies should be performed which are linked to concurrent comprehensive monitoring of overall STP performance. STPs with different treatment processes should be studied and the monitoring of full-scale plants should be accompanied by studies in pilot-plants and on laboratory scale.

Influence of advanced treatment processes on removal efficiencies

- Several advanced treatment processes have been investigated with the aim of reducing e.g. endocrine disrupters. Very high removal of > 90 % have been obtained with powdered activated carbon while removals from 40 to 70 % have been seen with magnetic ion exchange. Investigations of ozone treatment of an effluent from a municipal STP followed by UV treatment have shown promising results regarding the removal of endocrine disrupters.

Possible non-sewage related sources of estrogens to the aquatic environment

- Other sources to estrogens in the environment besides sewage effluent might be the outbringing of manure from life stock and sludge from sewage treatment plants. Too little is still known to assess their possible contribution via drain water to the total estrogenic activity in surface waters.

16 Konklusion (dansk)

Feminisering af hanfisk i ferskvandsmiljøet og det marine vandmiljø

- Feminisering af hanfisk er i dag blevet observeret i en række lande over hele verden. Denne form for hormonforstyrrelser er blevet set hos både en række ferskvands- og saltvandsfisk, skønt det oftest er set blandt ferskvandsarter.

- Feminiseringerne tilskrives en udledning af naturlige og syntetiske østrogener samt østrogene stoffer til det akvatiske miljø via spildevand.

- Tegnene på feminisering af hanfisk er generelt en produktion af blommeproteinet, vitellogenin - en markør for østrogen eksponering, samt udvikling af intersex, en unormal form for tvekønnethed, hvor hannerne udvikler ægceller i testiklerne.

- De værste tilfælde af feminisering, hvad angår både forekomst og grad af fænomenet, er set i England, mens en mindre hyppig udbredelse er set i øvrige lande – herunder Danmark.

- Man skal være opmærksom på, at kort-tids-eksponeringsforsøg for stofferne under mistanke ikke nødvendigvis giver et korrekt estimat af risikoen for vilde populationer af fisk, som lever hele deres liv i spildevandsbelastede vandløb.

De østrogene komponenter i spildevand

- En kombination af cellebaserede *in vitro* analysemetoder samt kemiske analyser af spildevand har verificeret og kvantificeret den østrogene aktivitet af spildevand i en række lande. Disse analyser har også demonstreret, at naturlige østrogener, 17ß-estradiol og østron, samt det syntetiske østrogen, ethinylestradiol, som anvendes i p-piller er sandsynlige kilder til nogle af de observerede forstyrrelser hos fisk fra spildevandsbelastede floder. I enkelte tilfælde er de østrogene kemikalier, alkylphenoler, også blevet antaget at være medansvarlige kandidater.

- I de fleste undersøgelser gør forekomsten af de nævnte stoffer fuldt ud rede for spildevandets samlede østrogenicitet.

Østrogenkoncentrationer i spildevand og overfladevand

- De tre naturlige østrogener, 17ß-estradiol, østron og østriol er globalt blevet detekteret henholdsvis i spildevand i koncentrationerne < 0,1 – 88 ng/l, (typisk 1 – 10 ng/l), < 0,1 – 220 ng/l (typisk 5 – 20) og < 0,1 – 42 ng/l. Ethinylestradiol er blevet fundet i koncentrationerne < 0,053 – 62 ng/l (typisk under 1 eller sjældent over 10 ng/l).

- I overfladevand er østrogenerne fundet i koncentrationerne 0.05 – 15.5 ng estradiol/l, < 0,1 – 17 ng estrone/l, < 0,1 – 3.4 ng estriol/l og < 0,053 – 30,8 ng ethinylestradiol/l med typiske koncentrationer under 5 ng/l for estradiol og østron og under 1 ng/l for ethinylestradiol.

Østrogeners skæbne i det vandige miljø

- Ethinylestradiol er mere persistent end de naturlige østrogener både i vand og sediment. Gennemsnitlige halveringstider for estradiol og østron i vand er blevet fastsat til henholdsvis 2,8 og 3,0 dage. Ethinylestradiol har en 10 gange så lang halveringstid som estradiol. I anaerobt sediment omdannes estradiol hurtigt til østron, men både østron og ethinylestradiol har meget lav nedbrydelighed i sediment og akkumulerer muligvis.

Forekomst og skæbne af alkylphenoler og bisphenol A i spildevand og overfladevand

- Nogle af de mere potente østrogene kemikalier, som kan udledes med spildevand, er alkylphenolerne, nonylphenol og octylphenol, samt bisphenol A. Nonylphenol og octylphenol er i spildevand generelt blevet fundet i koncentrationer under 10 µg/l og i overfladevand i koncentrationer under 1 µg/l, skønt der er få eksempler på koncentrationer over 300 µg/l i spildevand og 600 µg/l i overfladevand. Bisphenol A er sjældent fundet over 1 µg/l i hverken spildevand eller overfladevand. Både alkylphenoler og bisphenol A har et stort potentiale for at akkumulere i sedimentet.

Laveste effektkoncentrationer for feminisering med østrogener, alkylphenoler og bisphenol A

- Laboratorieforsøg har for 17ß-estradiol fundet en lavest effektkoncentration for induktion af vitellogenin på 5 ng/l og for induktion af intersex på 10 ng/l. En række andre effekter på det hanlige reproduktionssystem er set ved koncentrationer mellem 10 og 50 ng/l.

- Østron har samme eller lidt lavere østrogen potensitet sammenlignet med estadiol, og den laveste effekt koncentration for vitellogenin og intersex induktion hos hanner med østron er henholdsvis 30 og 10 ng/l. Der er kun lidt viden om potensiteten af østriol hos fisk men *in vivo* er det tilsyneladende 100 gange mindre østrogent end 17ß-estradiol.

- Ethinylestradiol er mere potent end de naturlige østrogener med hensyn til at fremkalde feminiserende effekter hos hanfisk. Vitellogeninproduktion og intersex i hanner er blevet induceret med 0,1 ng/l, og kønsratio er ændret mod hunner med en eksponering for 0,6 ng/l. En række andre testikeleffekter er set ved koncentrationer under 10 ng/l.

- Nonylphenol, oktylphenol og bisphenol A har en lavere østrogenicitet end de naturlige og syntetiske østrogener. Induktion af vitellogenin kan forekomme ved en eksponering for 5 µg/l nonylphenol eller octylphenol, og intersex, ændret kønsratio og andre effekter ved nonylphenol-koncentrationer mellem 30 og 100 µg/l. Forstyrrelser i det hanlig reproduktionssystem er set ved 2 µg/l oktylphenol. Bisphenol A kan fremkalde effekter ved koncentrationer mellem 10 og 40 µg/l.

Sammenhæng mellem lavest effektkoncentration for østrogenerne/østrogene stoffer og deres forekomst i vandmiljøet

- Sammenlignes spildevands- og overfladevandskoncentrationerne af østrogener, alkylphenoler og bisphenol A med den laveste effekt koncentration for reproduktionsforstyrrelser for de enkelte stoffer fremstår det, at koncentrationerne af østradiol, østron, ethinyløstradiol, nonylphenol og octylphenol i nogle tilfælde har været høje nok til at kunne mistænkes for at have fremkaldt feminisering af vilde populationer af fisk. Baseret på den nuværende viden omkring miljømæssig forekomst og

østrogene potentiale af østriol er det endnu ikke muligt at estimere bidraget af dette østrogen til de observerede feminiseringer. Bisphenol A er generelt blevet fundet i koncentrationer under den laveste effektkoncentration for inducering af reproduktive effekter.

- Hvilke stoffer, der er ansvarlige for den observerede feminisering af fisk i Danmark, er stadig uvist pga. begrænset viden om vandkoncentrationer af østrogener i de danske vandløb.

- Under en vurdering af den mulige betydning af østrogener/østrogene stoffer i miljøet på reproduktionssystemet hos fisk skal flere aspekter tage i betragtning. Østrogener og østrogene stoffer i spildevand vil virke additivt, hvorved man nedsætter den koncentration af et enkeltstof, som kræves for at fremkalde effekter. Forskellige arter udviser ikke samme sensitivitet. Timingen af eksponering i forhold til kritiske perioder i fiskens livscyklus er af stor betyding for de resulterende effekter, og gentagende eksponering for høje koncentrationer af østrogner ser ud til at give uforholdsmæssig store effekter.

Effekten af feminisering eller østrogen eksponering for han- og hunfisk's fertilitet

- Der er meget lidt viden om betydningen af de observerede tegn på feminisering for fertilitet og forplantningssucces hos fisk. Observationer blandt intersex skaller i England af en asynkron udvikling af kønsceller hos hanner og hunner pga en forsinket sædcelleudviling samt observationer af nedsat sædvolumen, sædcelledensitet og –motilitet har dog indikeret nedsat fertilitet. Den normale udvikling af ægceller hos hunner var også påvirket. Kontrollerede eksponeringsforsøg med de omtalte enkeltstoffer har også påvist nedsat befrugtningssucces blandt hanner og nedsat gydning af æg blandt hunner.

- Påvirkningen af østrogener og østrogene stoffer på reproduktionsevnen kan evt. også være af indirekte karakter via nedsatte energiresourcer til reproduktion.

Kilder til østrogener i spildevand

- Den pulje af østrogener, som når renseanlæggene stammer fra menneskets naturlige produktion af østrogener, fra hormon- og østrogenerstatningsterapi og fra indtagelsen af p-piller, som indeholder ethinyløstradiol.

- Østrogener udskillles hovedsageligt som vandopløselige konjugater. En estimering af østrogenekskretionen fra mennesker i Danmark har vist, at hovedparten af østrogenerne stammer fra den naturlige produktion, som bidrager med ca. 87 % af den totale ekskretion af østrogener. Ekskretionen fra hormonbehandling er ansvarlig for ca. 12 %, mens ekskretionen af hormoner fra p-piller udgør ca. 1 % af den totale mængde udskilt østrogen.

- Den totale udskillelelse pr. døgn af de fire østrogener er estimeret til 36 g østradiol, 69 g østron, 340 g østriol og 3,2 g ethinyløstradiol.

Kilder til alkylphenoler og bisphenol A i spildevand

- Alkylphenoler anvendes hovedsageligt i produktionen af andre produkter som f.eks. alkylphenolethoxylater. Alkylphenolethoxylater er relativt nemt nedbrudt til alkylphenoler og derfor vigtige kilder til alkylphenoler i

renseanlæg. Der forventes ikke nogen frigivelse af hverken alkylphenoler eller alkylphenolethoxylater fra produktion af sidstnævnte i Danmark.

- Nonylphenolfamilien repræsenterer ca. 85 % af alkylphenolmarkedet, og de resterende 15 % formodes at være octylphenol.

- Den totale frigivelse af nonylphenolethoxylater til spildevand i Danmark er estimeret til at være mellem 37 og 996 tons pr. år.

- Der antages ikke at foregå nogen produktion af bisphenol A i Danmark. Frigivelsen i Danmark forventes derfor udelukkende at stamme fra forarbejdning, brug og bortskaffelse af bisphenol A-holdige materialer. Frigivelsen af bisphenol A til spildevand i Danmark er estimeret til 17 kg pr. år fra PVC-bearbejdning, 256 kg fra PVC-anvendelse og 492 kg fra papirgenbrug.

Østrogeners skæbne i renseanlæg

- I tyske renseanlæg er det vist, at konjugerede steroider bidrager til ca. 50 % af steroiderne i indløbsvand og ca. 70 % af steroiderne i udløbsvand. Den totale mængde østrogener (østron, østradiol og ethinyløestradiol) var 25,5 ng/l og 9,3 ng/l i henholdsvis indløbs- og udløbsvand.

- Baseret på laboratorieforsøg forventes det, at konjugeret østradiol relativt hurtige dekonjugeres, og at østradiol oxideres til østron, som yderligere elimineres.

- Typiske gennemsnitlige fjernelseseffektiviteter for de fire østrogener fra renseanlæggenes vandfase er: 61 ± 38 % østron, 87 ± 9 % østradiol, 96 ± 6 % østriol og 85 ± 14 % ethinyløstradiol. Analyser af glucuronsyrekonjugater blev ikke inkluderet i de pågældende undersøgelser. Biologisk nedbrydning er den vigtigste proces for fjernelse af østrogener i renseanlæg. Reduktion i spildevandets østrogenicitet er også registreret ved primær (sorption til slampartikler) og tertiær rensning.

Alkylphenolers og bisphenol A's skæbne i renseanlæg

- De resulterende produkter fra nedbrydning af alkylphenolethoxylater under aerobe forhold i renseanlæggene er mono- og diethoxylater samt de mere hydrofobe carboxylater og alkylphenoler. Det endelige produkt, alkylphenol, ser ud til at være nedbrydeligt under aerobe forhold. Omdannelsen under anerobe forhold resulterer i produktionen af mono- og diethoxylater og endligt alkylphenoler. Koncentrationerne af alkylphenoler i anaerobt behandlet slam er ofte meget høje.

- Gennemsnitlige fjernelseseffektiviteter (vandfasen) for nonylphenolforbindelser på 53 % og 59 % er fundet ved undersøgelse af renseanlæg, hvor der er inkluderet både nonylphenolethoxylater og deres metabolitter incl. mono- og dicarboxylater.

- Der er såvidt vides ikke nogen undersøgelser af alkylphenolethoxylaters skæbne i renseanlæg, som inkluderer alle de kendte metabolitter af alkylphenolethoxylater.

- Bisphenol A forventes at blive nedbrudt nemt under aerobe forhold i renseanlæg men ikke under anaerobe eller anoxiske forhold. Desuden kan bisphenol A fjernes fra vandfasen ved sorption til slampartikler. Fjernelses

effektiviteter fra vandfasen på 47-96 % er blevet fundet i canadiske renseanlæg og 0-~96 % i danske lavteknologianlæg.

Renseanlægstypens betydning for fjernelsen af østrogener/østrogene stoffer

- Generelt ser fjernelseseffektiviteten for østrogener og alkylphenolforbindelser ud til at øges og spildevandskoncentrationerne som konsekvens heraf ud til at falde med stigende forbedring af renseanlæggene. For eksempel er en spildevandskoncentration på < 0,1-0,32 ng østradiol/l set i et californisk renseanlæg efter omvendt osmose.

- Anvendelse af lav-teknologi renseanlæg i såkaldt åben land kan resultere i relativt høje lokale koncentrationer af østrogener/østrogene stoffer i den akvatiske recipient.

- Forskellige drift forhold i renseanlæggene som vand- og slamtilbageholdelsestid, temperatur og belastningsgraden vil have en vigtig betydning for anlæggets effektivitet og dermed på fjernelsen af østrogener og xenoøstrogener. Der gives dog kun lidt information om driftforholdene i de undersøgelser af renseanlæg, som foreligger.

- Der er behov for mere viden om østrogener/ østrogene stoffers skæbne i renseanlæg. Der bør udføres højkvalitetsundersøgelser, som er forbundet med samtidig omfattende monitering af renseanlæggets overordnede ydeevne.

Betydningen af avancerede renseprocesser for fjernelseseffektiviteten

- Man har undersøgt adskillige avancerede renseprocesser med det mål at nedsætte udledelsen af hormonforstyrrende stoffer. Meget høj fjernelse på > 90 % er opnået med aktiveret kul, mens fjernelse fra 40 – 70 % er set vha. magnetisk ionbytning. Undersøgelser af ozonbehandling har vist lovende resultater angående fjernelse af hormonforstyrrende stoffer.

Mulige ikke-spildevandsrelaterede kilder til østrogener i det akvatiske miljø

- Andre kilder til østrogener i miljøet udover spildevand kan evt. være udbringelse af gylle fra husdyr og slam fra renseanlæg. Man ved dog endnu for lidt til at vurdere disse kilders mulige bidrag via drænvand til den østrogene aktivitet i overfladevand.

17 Reference List

1. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. Widespread sexual disruption in wild fish. *Environ Sci Technol* 32:2498-2506 (1998).

2. Christiansen LB, Plesner T. Intersex og andre effekter på reproduktionssystemet i skalle og bækørred – relationer til østrogener og østrogenlignende stoffer.Århus Amt,2001.

3. Larsson DGJ, Adolfsson-Erici M, Parkkonen J, Pettersson M, Berg AH, Olsson P-E, Förlin L. Ethinyloestradiol - an undesired fish contraceptive? *Aquat Toxicol* 45:91-97 (1999).

4. Knudsen FR, Schou AE, Wiborg ML, Mona E, Tollefsen K, Stenersen J, Sumpter JP. Increase of plasma vitellogenin concentration in rainbow trout (*Oncorhynchus mykiss*) exposed to effluents from oil refinery treatment works and municipal sewage. *Bull Environ Contam Toxicol* 59:802-806 (1997).

5. Hecker, M., Tyler, C. R., Hoffmann, M., Maddix, S., and Karbe, L. Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe river, Germany. *Environ Sci Technol* 36, 2311-2321. (2002).

6. Vethaak AD, Rijs GBJ, Schrap SM, Ruiter H, Gerritsen A, Lahr J. Estrogens and xeno-estrogens in the aquatic environment of the Netherlands. Occurrence, potency and biological effects. Riza/RikZ-report no. 2002.001.(2002).

7. Flammarion P, Brion F, Babut M, Garric J, Migeon B, Noury AP, Thybaud E. Induction of fish vitellogenin and alteration in testicular structure: Preliminary results of estrogenic effects in chub (*Leucicus cephalus*). *Ecotoxicol* 9:127-135 (2000).

8. Solé, M., Barceló, D., and Porte, C. Seasonal variation of plasmatic and hepatic vetellogenin and EROD activity in carp, *Cyprinus carpio*, in ralation to sewage treatment plants. *Aquat Toxicol.* (2002). In Press

9. Harshbarger JC, Coffey MJ, Young MY. Intersexes in Mississippi River shovelnose sturgeon sampled below Saint Louis Missouri, USA. *Marin Environ Research* 50:247-250 (2000).

10. Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette LJ. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (Cyprinus Carpio) captured near a major metropolitan sewage treatment plant. *Environ Health Perspect* 104:1096-1101 (1996).

11. Folmar LC, Denslow ND, Kroll K, Orlando EF, Enblom J, Marcino J, Metcalfe CD, Guillette LJ. Altered serum sex steroids and vitellogenin induction in walleye (*Stizostedion vitreum*) collected near a metropolitan

sewage treatment plant. *Arch Environ Contam Toxicol* 40:392-398 (2001).

12. Hashimoto S, Bessho H, Hara A, Nakamura M, Iguchi T, Fujita K. Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. *Mar Environ Research* 49:37-53 (2000).

13. Minier C, Caltot G, Leboulanger F, Hill EM. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex regions. *Analusis* 28:801-806 (2000).

14. Solé M, López de Alda MJ, Castillo M, Porte C, Ladegaard-Pedersen K, Barcelo D. Estrogenicity determination in sewage treatment plants and surface waters from the catalonian area (NE Spain). *Environ Sci Technol* 34:5076-5083 (2000).

15. van Aerle R, Nolan M, Jobling S, Christiansen LB, Sumpter JP, Tyler CR. Sexual disruption in a second species of wild cyprinid fish (the godgeon, *Gobio gobio*) in United Kingdom freshwaters. *Environ Toxicol Chem* 20:2841-2847 (2001).

16. Allen Y, Matthiessen P, Scott AP, Haworth S, Feist S, Thain JE. The extent of oestrogenic contamination in the UK estuarine and marine environments - further surveys of flounder. *Sci Tot Environ* 2333:5-20 (1999).

17. Allen Y, Scott AP, Matthiessen P, Haworth S, Thain JE, Feist S. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environ Toxicol Chem* 18:1791-1800 (1999).

18. Matthiessen, P., Allen, Y., Bamber, S., Craft, J., Hurst, M., Hutchinson, T., Feist, S., Katsiadaki, I., Kirby, M., Robinson, C., Scott, S., Thain, J., and Thomas, K. The impact of oestrogenic and androgenic contamination on marine organisms in the United Kingdom - summary of the EDMAR programme. *Marin Environ Research* . (2002) In Press

19. Minier C, Levy F, Rabel D, Bocquené G, Godefroy D, Borgeot T, Leboulenger F. Flounder health status in the Seine Bay. A multibiomarker study. *Marin Environ Research* 50:373-377 (2000).

20. Simpson MG, Parry M, Kleinkauf A, Swarbreck D, Walker P, Leah RT. Pathology of the liver, kidney and gonad of flounder (*Platichthys flesus*) from a UK estuary impacted by endocrine disrupting chemicals. *Mar Environ Research* 50:283-287 (2000).

21. Lye CM, Frid CL, Gill ME, McCormick D. Abnormalities in the reproductive health of flounder, *Platichthys flesus*, exposed to effluent from a sewage treatment works. *Mar Pollut Bull* 34:34-41 (1997).

22. Lye CM, Frid CL, Gill ME. Seasonal reproductive health of flounder *Platichthys flesus* exposed to sewage effluent. *Mar Ecol-Prog Ser* 170:249-260 (1998).

23. Kirby, M. F., Dyer, R. A., Matthiessen, P., Steve, F. W., Bignell, J. P., Jones, G. J., Craft, J. A., Brown, E., and Robinson, C. Morphologically intermediate papilla syndrome (MIPS) in UK populations of Sand Goby: Endocrine disruption ? Conference abstract from SETAC Europe 12th Annual Meeting 12-16 May 2002, Vienna, Austria (2002).

24. Rodgers-Gray TP, Jobling S, Morris S, Kelly C, Kirby S, Janbakhsh A, Harries JE, Waldock MJ, Tyler CR, Sumpter JP. Long-term temporal changes in the estrogenic compostion of treated sewage effluent and its biological effects on fish. *Mar Environ Research* 49:37-53 (2000).

25. Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. Identification of estrogenic chemicals in STW effluent. 1. chemical fractionation and in vitro biological screening. *Environ Sci Technol* 32:1549-1558 (1998).

26. Körner W, Spengler P, Bolz U, Schuller W, Hanf V, Metzger JW. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2.Biological analysis. *Environ Toxicol Chem* 20:2142-2151 (2001).

27. Körner W, Hanf V, Schuller W, Kempter C, Metzger J, Hagenmaier H. Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. *Sci Tot Environ* 225:33-48 (1999).

28. Körner W, Bolz U, Süßmuth W, Hiller G, Schuller W, Hanf V, Hagenmaier H. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. *Chemosphere* 40:1131-1142 (2000).

29. Shen JH, Gutendorf B, Vahl HH, Shen L, Westendorf J. Toxicological profile of pollutants in surface water from an area in Taihu Lake, Yangtze Delta. *Toxicology* 166:71-78 (2001).

30. Snyder SA, Villeneuve DL, Snyder EM, Giesy JP. Identification and quantification of estrogen receptor agonists in wastewater effluents. *Environ Sci Technol* 35:3620-3625 (2001).

31. Zillious EJ, Johnson IC, Kiparissis Y, Metcalfe CD, Wheat JV, Ward SG, Liu H. The sheepshead minnow as an *in vivo* model for endocrine disruption in marine teleosts: A partial life-cycle test with 17α-ethinylestradiol. *Environ Toxicol Chem* 20:1968-1978 (2001).

32. Murk AJ, Legler J, van Lipzig MMH, Meerman JHN, Belfroid AC, Spenkelink A, van der Burg B, Rijs GBJ, Vethaak D. Detection of estrogenic potency in wastewater and suface water with three *in vitro* bioassays. *Environ Toxicol Chem* 21:16-26 (2002).

33. Witters HE, Vangenechten C, Berckmans P. Detection of estrogenic activity in Flemish surface waters using an *in vitro* recombinant assay with yeast cells. *Water Sci Technol* 43:117-123 (2001).

34.Sheahan, D. A., Brighty, G. C., Daniel, M., Kirby, S. J., Hurst, M. R., Kennedy, M., Morris, S., Routledge, E. J., Sumpter, J. P, and Waldock,

M. J. estrogenic activity mwasured in a sewage treatment works treating industrial inputs containing high concentrtions of alkylphenolic compounds - a case study. *Environ Toxicol Chem* "![3], 507-514 (2002).

35. 35. Williams RJ, Johnson AC, Smith JJL, Jürgens MD, Holthaus K. Fate and behavior of steroid oestrogens in aquatic systems. R&D Technical Report P2-162/TR.Environment Agency, Bristol, England, (2001).

36. Jürgens MD, Holthaus KIE, Johnson AC, Smith JJL, Hetheridge M, Williams RJ. The potential for estradiol and ethinylestradiol degradation in English rivers. *Environ Toxico Chem* 21:480-488 (2002).

37. Young WF, Whitehouse P, Johnson I, Sorokin N. Proposed predicted-no-effelct-concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters. R&D Technical Report P2-T04/1.Environment Agency, Bristol, England, 2002.

38. Heinis LJ, Knuth ML, Liber K, Sheedy BR, Tunell RL, Ankley GT. Persistence and distribution of 4-nonylphenol following repeated application to littoral enclosures. *Environ Toxicol Chem* 18:363-375 (1999).

39. Voordeckers JW, Fennell DE, Jones K, Häggblom MM. Anaerobic biotransformation of tetrabromobisphenol A, tetrachlorobisphenol A, and bisphenol A in estuarine sediments. *Environ Sci Technol* 36:696-701 (2002).

40. Thorpe K, Hutchinson TH, Hetheridge M, Scholze M, Sumpter JP, Tyler CR. Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ Sci Technol* 35:2476-2481 (2001).

41. Thorpe KL, Hutchinson TH, Hetheridge MJ, Sumpter JP, Tyler CR. Development of an *in vivo* screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ Toxico Chem* 19:2818-2820 (2000).

42. Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughed RJ, Croley TR, March RE, Potter T. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with japanese medaka (*Oryzias latipes*). *Environ Toxicol Chem* 20:297-308 (2001).

43. Nimrod AC, Benson WH. Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. *Aquat Toxicol* 44:141-156 (1998).

44. Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. Effects of waterborne exposure of 17ß-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat Toxicol* 47:129-145 (1999).

45. Halm, S., Pounds, N., Maddix, S., Rand-Weaver, M., Sumpter, J. P, Hutchinson, T. H., and Tyler, C. R. Exposure to exogenous 17ß-oestradiol disrupts P450aromB mRNA expression in the brain and

gonad of adult fathead minnows (*Pimephales promelas*). *Aquac Toxicol* (2002). In Press

46. Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. *Environ Sci Technol* 32:1559-1565 (1998).

47. Panter GH, Thompson RS, Sumpter JP. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquat Toxiclo* 42:243-253 (1998).

48. Petersen GI, Norrgren.L., Holbech H, Lundgren A, Koivisto S. Suitablilty of zebrafish as testorganism for detection of endocrine disrupting chemicals. TemaNord 2001:597.Nordic Council of Ministers, (2001).

49. Knörr S, Braunbeck T. Decline in reproductive success, sex reversal, and developmental alterations in Japanese medaka (*Oryzias latipes*) after continuous exposure to octylphenol. *Ecotoxicol Environ Safety* 51:187-196 (2002).

50. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. Inhibition of Testicular Growth in Rainbow Trout (Oncorhynchus Mykiss) Exposed to Estrogenic Alkylphenolic Chemicals. *Environ Toxicol Chem* 15:194-202 (1996).

51. Schwaiger, J., Mallow, U., Knoerr, S., Braunbeck, T., Kalbfus, W., and Negele, R. D. How estrogenic in nonylphenol ? A transgenerationsal study using rainbow trout (*Oncorhynchus mykiss*) as a test organism. *Aquat Toxicol* (2002). In Press

52. Ackermann, G. E., Schwaiger, J., Negele, R. D., and Fent, K. Effects of long-term nonylphenol exposure on gonadal development and biomarkers of estrogen exposure in juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* (2002). In Press

53. Gray MA, Metcalfe CD. Induction of testis-ova in Japanese medake (*Oryzias latipes*) exposed to *p*-nonylphenol. *Environ Toxicol Chem* 16:1082-1086 (1997).

54. Miles-Richardson SR, Pierens SL, Nichols KM, Kramer VJ, Snyder EM, Render JA, Fitzgerald SD, Giesy JP. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of Fathead Minnows (*Pimephanles promelas*). *Environ Research Section* A 80:122-137 (1999).

55. Lindholst C, Pedersen KL, Pedersen SN. Estrogenic response of bisphenol A in rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* 48:87-94 (2000).

56. Sohoni P, Tyler CR, Hurd K, Caunter J, Hetheridge M, Williams T, Woods C, Evans M, Toy R, Garcas M. Reproductive effects of long-term exposure to bisphenol A in the rathead minnow (*Pimephales promelas*). *Environ Sci Technol* 35:2917-2925 (2001).

57. The identification of oestrogenic effects in wild fish - Phase II. R & D Technical Report W2-014/TR.Environment Agency, Bristol, UK, (2002).

58. Piferrer F. Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture* 197:229-281 (2001).

59. Panter GH, Thompson RS, Sumpter JP. Intermittent exposure of fish to estradiol. *Environ Sci Technol* 34:2756-2760 (2000).

60. Jobling S, Beresford N, Nolan M, Rodgers-Gray TP, Brighty G, Sumpter JP, Tyler CR. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol Reprod* 66:272-281 (2002).

61. Van den Belt K, Verheyen R, Witters H. Reproductive effects of ethynylestradiol and 4*t*-octylphenol on the zebrafish (*Danio rerio*). *Arch Environ Contam Toxicol* 41:458-467 (2001).

62. Shioda T, Wakabayashi M. Effect of certain chemicals on the reproduction of medaka (*Oryzias latipes*). *Chemosphere* 40:239-243 (2000).

63. Shioda T, Wakabayashi M. Evaluation of reproductivity of medaka (*Oryzias latipes*) exposed to chmicals using a 2-week reproduction test. *Water Sci Technol* 42:53-60 (2000).

64. Herman RL, Kincaid HL. Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* 51:431-441 (1988).

65. Folmar LC, Gardner GR, Schreibman MP, Magliulo-Cepriano L, Mills LJ, Zaroogian G, Gutjahr-Gobell R, Haebler R, Horowitz DB, Denslow N. Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). *Aquat Toxicol* 51:431-441 (2001).

66. Sheahan DA, Bucke D, Matthiessen P, Sumpter JP, Kirby MF, Neall P, Waldock M. The effects of low level 17α-ethinylestradiol upon plasma vitellogenin levels in male and female rainbow trout, *Oncorhynchus mykiss* held at two acclimation temperatures. In: Sublethal and chronic effects of pollutants on freshwater fish (Muller R, Lloyd R, eds).Fishing News Books;99-112, (1993).

67. Schwaiger J, Spieser OH, Bauer C, Ferling H, Mallow U, Kalbfus W, Negele RD. Chronic toxicity of nonylphenol and ethinylestradiol: haematological and histopathological effects in juvenile Common carp (*Cyprinus carpio*). *Aquat Toxicol* 51:69-78 (2000).

68. Örn S, Gessbo Å, Steinholz Å, Norrgren.L. Zebrafish (*Danio rerio*) - A candidate to evaluate endocrine disrupting chemicals. In: TemaNord 555. Zebra Fish for Testing Endocrine Disrupting Chemicals (2000).

69. Harris CA, Santos EM, Janbakhsh A, Pottinger TG, Tyler CR, Sumpter JP. Nonylphenol affects gonadotropin levels in the pituitary gland and plasma of female rainbow trout. *Environ Sci Technol* 35:2909-2916 (2001).

70. Kuhl H. Pharmacokinetics of oestrogens and progestogens. *Maturitas* 12:171-197 (1990).

71. Schubert W, Cullberg G, Edgar B, Hedner T. Inhibition of 17β-estradiol metabolism by grapefruit juice in ovariectomized women. *Maturitas* 20:155-163 (1995).

72. Johnson AC, Belfroid A, Di Corcia A. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. *Sci Tot Environ* 256: (2000).

73. Frandsen VA. *Acta indocr* 53:93-100 (1966).

74. Vander AJ, Sherman.J.H., Luciano DS. *Human physiology*. New York:McGraww-Hill Inc., (2002).

75. Danmarks Statistik. Statistisk årbog 2001.Danmarks Statistik, (2001).

76. EU. Risk assessment of 4-nonylphenol (branched) and nonylphenol, Draft.EU, (1999).

77. Alkylphenol research council. Alkylphenol research council homepage. Available: http://www.aperc.org [cited 5 July 2002].

78. Nimrod AC, Benson WH. Environmental estrogenic effects of alkylphenol ethoxylates. *Cril Rev Toxicol* 26:335-364 (1996).

79. Bisphenol A Industry. Bisphenol A hompage. Available: http://www.bisphenol-a.org [cited 8 July 2 A.D.].

80. Ternes, T. A., Kreckel, P., and Mueller, J. Behaviour and occurrence of estrogens in municipal sewage treatment plants -- II. Aerobic batch experiments with activated sludge. The Science of the Total Environment 225[1-2] (1999).

81. Panter, Grace H., Thompson, Roy S., Beresford, Nicola, and Sumpter, John P. Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. Chemosphere 38[15] (1999).

82. Adler P, Steger-Hartmann T, Kalbfus W. Vorkommen natürlicher und synthetischer östrogener steroide in wässern des süd unde mittelseutschen raumes. *Acta Hydrochim Hydrobiol* 29:227-241 (2001).

83. Winther-Nielsen W, Madsen T. Biologisk nedbrydning af østradiol.Vandkvalitetsinstituttet, Hørsholm., (1996).

84.Layton, A. C., Gregory, B. W., Seward, J. R., Schultz, T. W., and Sayler, G. S. Mineralization of Steroidal Hormones by Biosolids in Wastewater Treatment Systems in Tennessee U.S.A. *Environ Sci Technol* - Columbus 34[18] (2000).

85. 85. Lee, Hing Biu and Peart, Thomas E. Residues And Trace Elements - Determination of 17b-Estradiol and Its Metabolites in Sewage Effluent by Solid-Phase Extraction and Gas Chromatography/Mass Spectrometry. *J AOAC Int* 81[6] (1998).

86. Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R. D., and Servos, M. Behavior and occurrence of estrogens in municipal sewage treatment plants -- I. Investigations in Germany, Canada and Brazil. *Sci Tot Environ* 225[1-2] (1999).

87. Baronti, Chiara, Curini, Roberta, Ascenzo, Giuseppe D', Corcia, Antonio Di, Gentili, Alessandra, and Samperi, Roberto. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ Sci Technol* - Columbus 34[24] (2000).

88. Johnson, A. C., Belfroid, A., and Di Corcia, A. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. *Sci Tot Environ* 256[2-3] (2000).

89. Nasu, M., Goto, M., Kato, H., Oshima, Y., and Tanaka, H. Hazard assessment and control of environmental contaminants - Study on endocrine disrupting chemicals in wastewater treatment plants. *Water Sci Technol* 43[2] (2001).

90. Staples, Charles A., Naylor, Carter G., Williams, James B., and Gledhill, William E. Ultimate biodegradation of alkylphenol ethoxylate surfactants and their biodegradation intermediates. *Environ Toxicol Chem* 20[11] (2001).

91. Ejlertsson, Jorgen, Nilsson, Marie Louise, Kylin, Henrik, Bergman, Ake, Karlson, Leif, Oquist, Mats, and Svensson, Bo H. Anaerobic Degradation of Nonylphenol Mono- and Diethoxylates in Digestor Sludge, Landfilled Municipal Solid Waste, and Landfilled Sludge. *Environ Sci Technol* - Columbus 33[2] (1999).

92. Knudsen L., Kristensen G.H., Jørgensen P.E., Jepsen S.-E. Reduction of the content of organic micropollutants in digested sludge by a post-aeration process - a full-scales demonstration. *Water Sci Technol* 42:111-118 (2000).

93. Ahel, Marijan, Giger, Walter, and Koch, Markus. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment--I. Occurrence and transformation in sewage treatment. *Water Research* 28[5] (1994).

94. Lee, H. B. and Peart, T. E. Occurrence and elimination of nonylphenol ethoxylates and metabolites in municipal wastewater and effluents. *Water Qual Res J Can* 33[3] (1998).

95. Johnson, A. C. and Sumpter, J. P. Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environ Sci Technol* - Columbus 35[24] (2001).

96. Lee, Hing Biu and Peart, Thomas E. Environmental Analysis - Determination of bisphenol A in sewage effluent and sludge by solid-phase and supercritical fluid extraction and gas chromatography/mass spectrometry. *J AOAC Int* 83[2] (2000).

97. Huang, C. H. and Sedlak, D. L. Environmental Chemistry - Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent asssay and gas chromatography/tandem mass spectrometry. *Environ Toxicol Chem* 20[1] (2001).

98. Boutrup S, Plesner T. Miljøfremmede stoffer i Århus amt 1998-2001. Spildevand, overfladisk afstrømning fra befæstede arealer og atmosfærisk deposition.Århus amt, natur og miljø, (2001).

99. Christiansen LB, Plesner T. Intersex og andre effekter på reproduktionssystemet i skaller og bækørred- relationer til østrogener og østrogenlignende stoffer.Århus amt, natur og miljø, (2001).

100. Shishida, K., Echigo, S., Kosaka, K., Tabasaki, M., Matsuda, T., Takigami, H., Yamada, H., Shimizu, Y., and Matsui, S. Evaluation of advanced sewage treatment processes for reuse of wastewater using bioassays. *Environ Technol* - London 21[5] (2000).

101. Walker, D. Oestrogenicity and wastewater recycling: experience from Essex and Suffolk water. Water and environmental management [14], 427-431 (2000).

102. Schäfer AI, Waite TD. Removal of endocrine disruptors in advanced treatment- the Australian approach. IWA workshop on endocrine disruptors, 9th april (2002).

103. Ried A, Mielcke J. Integrated ozone and UV applications for additional waste water treatment, disinfection and degradation of endocrine substances. IOA conference 15-16 april 2002, HongKong., 15 June (2002).

104. Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge EJ, Rycroft R, Sumpter JP, Tylor T. A survey of estrogenic activity in United Kingdom inland waters. *Environ Toxicol Chem* 15:1993-2002 (1996).

105. Mueller GC, Kim U-H. Displacement of estradiol from estrogen receptors by simple alkyl phenols. *Endocrinology* 102:1429-1435 (1978).

106. Sharpe R, Skakkebaek NE. are oestrogens involved in falling sperm counts and dieorders of the male reproductive tract ? *Lancet* 341:1392-1395 (1993).

107. Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, Tylor T, Zaman N. Estrogenic activity in five united kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem* 16:534-542 (1997).

108. Mommsen TP, Walsh PJ. Vitellogenisis and oocyte assembly. In: Fish Physiology, Vol XIA (1988);348-405.

109. Henderson NE. The annual cycle in the testis of the eastern brook trout, *Salvelinus fontinalis* (Mitchill). *Can J Zool* 40:631-645 (1962).

110. Russel LD, Griswold MD. The sertoli cell. Cache River Press, (1993).

111. Nagler JJ, Bouma J, Thorgaard GH, Dauble DD. High incidence of a male-specific genetic marker in phenotypic female Chinnook Salmon form the Columbia River. *Environ Health Perspect* 109:67-69 (2001).

112. Noaksson E, Tjärnlund U, Bosveld TC, Balk L. Evidence for endocrine disruption in perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) in a remote Swedish lake in the vicinity of a public refuse dump. *Toxicol Appl Pharmacol* 174:160-176 (2001).

113. Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock M, Sumpter JP, Tyler CR. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environ Sci Technol* 35:462-470 (2001).

114. Hansen P-D, Dizer H, Hock B, Marx A, Sherry J, McMaster J, Blaise C. Vitellogenin - a biomarker for endocrine disruptors. *Trends Anal Chem* 17:448-451 (1998).

115. Howell WM, Black DA, Bortone SA. Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: Evidence for environmentally-induced masculinization. *Copeia* 4:676-681 (1980).

116. Legler, J., Spenkelink, B., De Voogt, P., Leonards, P., Vethaak, A. D., Schrap, M., and Murk, A. J. Measurement of estrogenic and dioxin-like activity in the Dutch aquatic environment with *in vitro* reporter gene assays. SETAC Europ 12th Annual Meeting.12-16 May, 2002, Vienna, Austia (2002).

117. Johnson AC, Sumpter JP. Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environ Sci Technol* 35:4697-4703 (2001).

118. Panter GH, Thompson RS, Beresford N, Sumpter JP. Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. *Chemosphere* 38:3579-3596 (1999).

119. Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. Estrogenic effects of effluents from sewage treatment works. *Chem Ecol* 8:275-285 (1994).

120. Hemming JM, Waller WT, Chow MC, Denslow ND, Venables B. Assessment of the estrogenicity and toxocity of a domestic watewater effluent flowing through a constructed wetland system uding biomarkers in male fathead minnows (*Pimphales promelas* Rafinesque, 1820). *Environ Toxicol Chem* 20:2268-2275 (2001).

121. Nichols KM, Miles-Richardson SR, Snyder EM, Giesy JP. Effects of exposure to minicipal wastewater in situ on the reproductive physiology of the fathead minnow (*Pimephales promelas*). *Environ Toxico Chem* 18:2001-2012 (1999).

122. Bjerregaard P, Korsgaard B, Christiansen LB, Pedersen KL, Christensen LJ, Pedersen SN, Horn P. Monitoring and risk assessment

for endocrine disrupters in the aquatic environment: A biomarker approach. *Arch Toxicol Suppl* 20:97-107 (1998).

123. Rasmussen U, Rosenkilde P, Jacobsen BN, Kristensen BI. Spildevand indeholder østrogene stoffer. *Vand og jord* 7:68-70 (2000).

124. Povlsen A. Hormonforstyrrence effekter af spildevand på regnbueørred (*Oncorhynchus mykiss*) Specialerapport, Syddansk Universitet 2001.

125. Orn, S., Borg, D., Holbech, H., Norman, A., Gessbo, A., and Norrgren.L. Short-term exposure of zebrafish to effluents from municipal sewage treatment woks and pulp mills. SETAC Europ 12th Annual Meeting.12-16 May, 2002, Vienna, Austia . 2002.

126. Bortone SA, Davis WB. Fish intersexuality as an indicator of environmental stress. *Bioscience* 44:165-172 (1994).

127. Marx, A., Sherry, J., Hansen, P.-D., and Hock, B. A new monoclonal antibody against vitellogenin from rainbow trout (*Oncorhynchus mykiss*). *Chemosphere* 44, 393-399 (2001).

128. Gimeno S, Gerritsen A, Bowmer T. Feminization of male carp. *Nature* 384:221-222 (1996).

129. Thomas KV, Hurst MR, Matthiessen P, Waldock MJ. Charachterization of estrogenic compounds in water samples collected from United Kingdom estuaries. *Environ Toxicol Chem* 20:2165-2170 (2001).

130. Aagaard T. 3,4,3',4'-tetrachlorobiphenyl and 17β-østradiols effekt på vitellogeninsyntesen hos skrubben (*Platichthys flesus).* Specialerapport. Biologisk Institut, Syddansk Universitet (2000).

131. Oh S-M, Choung S-Y, Sheen Y-Y, Chung K-H. Quantitative assessment of estrogenic activity in the wqater environment of Korea by the E-Screen assay. *Sci Tot Environ* 263:161-169 (2000).

132. Tollefsen, K. E., Byberg, A. L., Kelly, A., Grung, M., Utvik, T. I. R., and Goksøyr, A. Detection of estrogens and antiestrogens in effluents from oil production activities - A TiE approach. SETAC Europ 12th Annual Meeting.12-16 May, 2002, Vienna, Austia (2002).

133. Kime DE. A strategy for assessing the effects of xenobiotics on fish reproduction. *Sci Tot Environ* 225:3-11 (1999).

134. Pawlowski, S., Schmidt, T., Ternes, T., Kluczka, T., Nau, H., Erdinger, L., and Braunbeck, T. Combined *in situ* and *in vitro* experiments to assess estrogenic activity of environmental water samples. SETAC Europ 12th Annual Meeting.12-16 May, 2002, Vienna, Austia (2002).

135. Behnisch PA, Fufii K, Shiozaki K, Dawakami I, Sakai S. Estrogenic and dioxin-like potency in each step of a controlled landfill leachate treatment plant in Japan. *Chemosphere* 43:977-984 (2001).

136. Balaguer P, Fenet H, Georget V, Comunale F, Térouanne B, Gilbin R, Gomes E, Boussioux A-M, Sultan C, Pons M, Nicolas J-C, Casellas C.

Reporter cell lines to monitor steroid and antisteroid potential of environmental samples. *Ecotoxicology* 9:105-114 (2000).

137. Arijs, K., Verslycke, T., Vandenbergh, G. F., Slabbert, J. L., and Janssen, C. R. Estrogenic activity in South African inland waters asessed by a yeast estrogenic screen. SETAC Europ 12th Annual Meeting.12-16 May, 2002, Vienna, Austia (2002).

138. Kuch HM, Ballschmiter K. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. *Environ Sci Technol* 35:3201-3206 (2001).

139. Xiao X-Y, McCalley DV, Mcevoy J. Analysis of estrogens in river water and effluents using solid-phase extraction and gas chromatography-negative chemical ionisation mass spectrometry of the pentafluorobenzoyl derivatives. *J Chrom A* 923:195-204 (2001).

140. Johnson AC, Belfroid AC, Di Corcia A. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. *Sci Tot Environ* 256:163-173 (2000)

141. Andersen H, Ternes T, Halling-Sørensen B. Steroidøstrogener i renset spildevand kan være årsag til effekter på danske fisk. *Dansk Kemi* 83: (2002).

142. Niven SJ, Snape J, Hetheridge M, Evans M, Mcevoy J, Sutton PG, Rowland SJ. Investigations of the origins of estrogenic A-ring aromatic steroids in UK sewage treatment works effluents. *The Analyst* 126:285-287 (2001).

143. Stumpf M, Ternes TA, Haberer K, Baumann W. Nachweis von natürlichen und synthetischen östrogenen in klläranlagen und fließgewässern. *Vom Wasser* 87:251-261 (1996).

144. Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken R-D, Servos M. Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil. *Sci Tot Environ* 225:81-90 (1999).

145. Belfroid AC, van der Horst A, Vethaak AD, Schäfer AJ, Rijs GBJ, Wegener J, Cofino WP. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci Tot Environ* 225:101-108 (1999).

146. Baroti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R. Monitoring natural and synthetic estrognes at activated sludge sewage treatment plants and in a receiving river water. *Environ Sci Technol* 34:5059-5066 (2000).

147. Snyder SA, Keith TL, Verbrugge DA, Snyder EM, Gross TS, Kannan K, Giesy JP. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ Sci Technol* 33:2814-2820 (1999).

148. Huang C-H, Sedlak DL. Analysis of estrogenogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem spectrometry. *Environ Toxicol Chem* 20:133-139 (2001).

149. Lee H-B, Peart TE. Determination of 17ß-estradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. *J AOAC Int* 81:1209-1216 (1998).

150. Lai KM, Johnson KL, Scrimshaw MD, Lester JN. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. *Environ Sci Technol* 34:3890-3894 (2000).

151. Peck, M., Gibson, R., Kortenkamp, A., and Hill, E. Profiles of estrogenic contaminants present in effluents and sediments of two UK rivers. SETAC Europ 12th Annual Meeting.12-16 May, 2002, Vienna, Austia (2002).

152. Blackburn MA, Waldock MJ. Concentrations of Alkylphenols in Rivers and Estuaries in England and Wales. Water Res 29:1623-1629 (1995).

153. Hale RC, Smith CL, de Fur PO, Harvey E, Bush WO, La Guardia MJ, Vadas GG. Nonylphenols in sediments and effluents associated with diverse wastewater outfalls. *Environ Toxicol Chem* 19:946-952 (2000).

154. Rudel RA, Melly SJ, Geno PW, Sun G, Brody JG. Identification of alkylphenols and other estrogenic phenolic compounds in watewater, septage, and groundwater on cape cod, Massachusetts. *Environ Sci Technol* 32:861-869 (1998).

155. Ahel M, Giger W, Schaffner C. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment - II. Occurrence and transformation in rivers. *Wat Res* 28:1143-1152 (1994).

156. Vandløb og kilder 2000. NOVA 2003. DMU Report nr. 378:2002.

157. Heemken OP, Reincke H, Stachel B, Theobald N. The occurrence of xenoestrogens in the Elbe river and the North Sea. *Chemosphere* 45:245-259 (2001).

158. Bolz U, Hagenmaier H, Körner W. Phenolic xenoestrogens in surface water, sediments, and sewage sludge from Baden-Württemberg, south-west Germany. *Environ Pollut* 115:291-301 (2001).

159. Bennie DT, Sullivan CA, Lee H-B, Peart TE, Maguire RJ. Occurrence of alkylphenols and alkylphenol mono- and diethoxylates in natural waters of the Laurentian Great Lakes basin and the upper St. Lawrence River. *Sci Tot Environ* 193:263-275 (1997).

160. Naylor CG, Mieure JP, Adams WJ, Weeks JA, Castaldi FJ, Ogle LD, Romano RR. Alkylphenol ethoxylates in the environment. *JAOCS* 69:695-703 (1992).

161. Johnson AC, White C, Bharway L, Jürgens MD. Potential for octylphenol ato biodegrade in some English rivers. *Environ Toxicol Chem* 19:2486-2492 (2000).

162. Staples CA, Naylor CG, Williams JB, Gledhill WE. Ultimate biodegradation of alkylphenol ethoxylate surfantants and their biodegradation intermediates. *Environ Toxicol Chem* 20:2450-2455 (2001).

163. Staples CA, Williams JB, Blessing RL, Varineau PT. Measuring the biodegradability of nonylphenol ether carboxylates, octylphenol ether carbowoyates, and nonylphenol. *Chemosphere* 38:2029-2039 (1999).

164. Johnson AC, White C, Besien TJ, Jürgens MD. The sorption potential of octylphenol, a xenobiotic oestrogen, to suspended and bed-sediments collected from industrial and rural reaches of three English rivers. *Sci Tot Environ* 210/211:271-282 (1998).

165. Marcomini A, Pojana G, Sfriso A, Alonso J-MQ. Behaviour of anionic and nonionic surfactants and their persistent metabolites in the Venice Lagoon, Italy. *Environ Toxicol Chem* 19:2000-2007 (2000).

166. Ahel M, Giger W. Aqueous Solubility of Alkylphenols and Alkylphenol Polyethoxylates. CHEMOSPHERE 26:1461-1470 (1993).

167. Khim JS, Kannan K, Villeneuve DL, Koh CH, Giesy JP. Characterization and distribution of trace organic contaminants in sediment from Masan Bay, Korea. 1. Instrumental analysis. *Environ Sci Technol* 33:4205 (1999).

168. Lye CM, Frid CL, Gill ME, Cooper DW, Jones DM. Estrogenic alkylphenols in fish tissues, sediments, and waters from the U.K. Tyne and Tees estuaries. *Environ Sci Technol* 33:1009-1014 (1999).

169. Fromme H, Küchler T, Otto T, Pilz K, Müller J, Wenzel A. Occurrence of phthalates and bisphenol A and F in the environment. *Water Research* 36:1429-1438 (2002).

170. Lee H-B, Peart TE. Bisphenol a contamination in Canadian municipal and industrial wastewater and sludge samples. *Water Qual Res J Canada* 35:283-298 (2000).

171. Chemical substances in the environment. Japanese Environment Agency,1997.

172. Staples CA, Dorn PB, Klecka GM, O'Block ST, Branson DR, Harris LR. Bisphenol A concentrations in receiving waters near US manufacturing and processing facilities. *Chemosphere* 40:521-525 (2000).

173. Staples CA, Dorn PB, Klecka GM, O´Block ST, Harris LR. A review of the environmental fate, effects, and exposures of bisphenol A. *Chemosphere* 36:2149-2173 (1998).

174. Klecka GM, Gonsior SJ, West RJ, Goodwin PA, Markham DA. Biodegradation of bisphenol A in aquatic environments: river die-away. *Environ Toxicol Chem* 20:2725-2735 (2001).

175. Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. Some alkyl hydroxy benzoate preservatives (parabens) are oestrogenic. *Toxicol Appl Pharmacol* 153:12-19 (1998).

176. Iwamatsu T. Convenient method for sex reversal in a freshwater teleost, the medaka. *J Exp Biol* 283:210-214 (1999).

177. Christiansen T, Korsgaard B, Jespersen Å. Effects of nonylphenol and 17ß-oestradiol on vitellogenin synthesis, testicular structure and cytology in male eelpout (*Zoarces viviparus*). *J Exp Biol* 201:179-192 (1998).

178. Bjerselius R, Lundstedt-Enkel K, Olsén H, Mayer I, Dimberg K. Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17ß-estradiol. *Aquac Toxicol* 53:139-152 (2001).

179. Islinger, M., Willimski, D., Völkl, A., and Braunbeck, T. Effects of 17a-ethinylestradiol on the expression of three estrogen-responsive genes and cellular ultrastructure of liver and testes in male zebrafish. *Aquat Toxicol* (2002).

180. Allner B, Wegener G, Knacker T, Stahlschmidt-Allner P. Electrophoretic determination of estrogen-induced protein in fish exposed to synthetic and naturally occurring chemicals. *Sci Tot Environ* 233:21-31 (1999).

181. Rose J, Holbech H, Lindholst C, Nørum U, Povlsen A, Korsgaard B, Bjerregaard P. Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish (*Danio rerio*). *Com Biochem Physiol Part* C 131:531-539 (2002).

182. Van den Belt K, Wester PW, van der Ven LTM, Verheyen R, Witters H. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): Time dependency and reversibility. *Environ Toxicol Chem* 21:767-775 (2002).

183. Scholz S, Gutzeit HO. 17-α-ethinylestradiol affects rerpoduction, sexual differentiation and aromatse gene expression of the medaka (*Oryzias latipes*). *Aquat Toxicol* 50:363-373 (2000).

184. Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1216-1227 (2001).

185. Papoulis DM, Noltie DB, Tillitt DE. An *in vivo* model fish system to test chemical effects on sexual differentiation and development: exposuure to ethinyl estradiol. *Aquat Toxicol* 48:37-50 (1999).

186. Hemmer MJ, Hemmer BL, Bowman CJ, Kroll KJ, Folmar LC, Marcovich D, Hoglund MD, Denslow ND. Effects of p-nonylphenol, methoxychlor, and endosulfan on vitellogenin induction and expression in sheepshead minnow (*Cyprinodon variegatus*). *Environ Toxicol Chem* 20:336-343 (2001).

187. Giesy JP, Pierens SL, Snyder EM, Miles-Richardson SR, Kramer VJ, Snyder SA, Nichols KM, Villeneuve DL. Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). *Environ Toxicol Chem* 19:1368-1377 (2000).

188. Weber LP, Kiparissis Y, Hwang GS, Niimi AJ, Janz DM, Metcalfe CD. Increased cellular apoptosis after chronic aquaeous exposure to nonylphenol and quercetin in adult medaka (*Oryzias latipes*). *Comp Biochem Physiol C* 131:51-59 (2002).

189. Kwak H-I, Bae M-O, Lee M-H, Lee Y-S, Lee B-J, Kang KS, Chae C-H, Sung H-J, Shin J-S, Kim J-H, Mar.W.-C., Sheen Y-Y, Cho M-H. Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordtail fish (*Xiphophorus helleri*). *Environ Toxicol Chem* 20:787-795 (2001).

190. Pedersen SN, Christiansen LB, Pedersen KL, Korsgaard B, Bjerregaard P. *In vivo* estrogenic activity of branched and linear alkylphenols in rainbow trout (*Oncorhynchus mykiss*). *Sci Tot Environ* 233:89-96 (1999).

191. Gronen S, Denslow N, Manning S, Barnes S, Barnes D, Brouwer M. Serum vitellogenin levels and reproductive impairment of male japanese medaka (*Oryzias latipes*) exposed to 4-*tert*-octylphenol. *Environ Health Perspect* 107:385-390 (1999).

192. Gray MA, Niimi AJ, Metcalfe CD. Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. *Environ Toxicol Chem* 18:1835-1842 (1999).

193. Ahel M, Giger W, Koch M. Behavior of Alkylphenol Polyethoxylate Surfactants in the Aquatic Environment .1. Occurrence and Transformation in Sewage Treatment. *Water Res* 28:1131-1142 (1994).

194. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135:175-182 (1994).

195. Routledge EJ, Sumpter JP. Estrogenic Activity of Surfactants and Some of Their Degradation Products Assessed Using a Recombinant Yeast Screen. *Environ Toxicol Chem* 15:241-248 (1996).

196. Jobling S, Sumpter JP. Detergent Components in Sewage Effluent Are Weakly Estrogenic to Fish An in Vitro Study Using Rainbow Trout (Oncorhynchus Mykiss) Hepatocytes. *Aquat Toxicol* 27:361-372 (1993).

197. Wine RN, Li L-H, Barnes L.H., Gulati DK, Chapin RE. Reproductive toxicity of di-*n*-butylphthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ Health Perspect* 105:102-107 (1997).

198. Li L-H, Jester WF, Orth JM. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured sertoli cells and gonocytes from neonatal rats. *Toxicol Appl Pharmacol* 153:258-265 (1998).

199. Harris CA, Henttu P, Parker MG, Sumpter JP. The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* 105:802-811 (1997).

200. Sohoni P, Sumpter JP. Several environmental oestrogens are also anti-androgens. *J Endocrinol* 158:327-339 (1998).

201. Korsgaard, B., Andreassen, T. K., and Rasmussen, T. H. Effects of an environmental estrogen, 17α-ethinyl-estradiol, on the maternal-fetal trophic relationship in the eelpout *Zoarces viviparus* (L). *Mar Environ Res* (2002).

202. Schweinfurth H, Länge R, Gunzel P. Environmental fate and ecological effects of steroidal estrogens. IBC Conference Proocedings of "Oestrogenic chemicals in the Environment", London, 9-10 maj (1996).

203. Kramer VJ, Miles-Richardson SR, Pierens SL, Giesy JP. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17ß-estradiol. *Aquat Toxicol* 40:225-360 (1998).

204. Reis-Henriques MA, Cruz MM, Pereira JO. The modulating effect of vitellogenin on the synthesis of 17ß-estradiol by rainbow trout (*Oncorhynchus mykiss*) ovary. *Fish Physiol Biochem* 16:181-186 (1997).

205. Lycos. WebMD. Available: http://webmd.lycos.com/encyclopedia/; [cited 11 July 2 A.D.].

206. Bartsch W. Interrelationships between sex hormone-binding globulin and testoosterone, 5α-dihydrotestosterone and oestradiol-17β in blood of normal men. *Maturitas* 2:109-118 (1980).

207. Brown JB. *Lancet* 1:320-323 (1955).

208. Fotherby K. Methabolism of synthetic steroids by animals and man. *Acta indocr* 119-147 (1974).

209. Aldercreutz H. *J Steroid Biochem* 17:639-645 (1982).

210. Barnes RB. Menopause: physiology and pharmacology.Year book medical publishers, (1987).

211. Aldercreutz H. *J Steroid Biochem* 13:231-244 (1980).

212. Fotherby K. *Meth Find Exptl Clin Pharmacol* 4:133-141 (1982).

213. Thybo H, Manniche V. P-pillens fordele og ulemper. Available: http://www.sundhed.dk/ [cited 22 July 2002].

214. Sundhed-dk. Medicinhåndbogen hos sundhed.dk. Available: www.sundhed.dk.

215. Danish Medicines Agency. Medicinal products statistics 1996-2000.Danish Medicines Agency,2001.

216. Setchell KDR. Naturally occurring non-steroidal estrogens of dietary origin. In: Estrogens in the environment (McLachlan JA, ed).Elsevier Science Publishing Co, Inc.,1985;69-84.

217. Sasol. Sasol homepage. Available: http://www.condea.com/products/alkylphenoles/end_use.asp [cited 5 July 2002].

218. Nordic Counsel of Ministers. Chemicals with estrogen like effects.Nordic council of ministers, (1996).

219. Rasmussen JO, Madsen T, Skak C. Substitution af alkylphenolethoxylater (APE) i maling, træbeskyttelse, lime og fugemasser, Fase 1.Miljøstyrelsen, Miljø og Energiministeriet, (2002).

220. Vikelsøe J, Thomsen M, Johansen E. Sources of phthalates and nonylphenoles in municipal waste water. Neri technical report no 225.Ministry of Environment and Energy, (1998).

221. EU. Risk assessment of Bisphenol-A, Draft. ECB, Internet: 2002.

222. Veirup B. Telephone conversation with Birgitte Veirup, Plastindustrien i Danmark. Personal communication.

223. Grøn information. Grøn informations hjemmeside. Available: http://www.greeninfo.dk [cited 15 July 2002].

224. PVC informationsrådet. PVC informationsrådets hjemmeside. Available: www.pvc.dk [cited 15 July 2002].

225. Xerox. Xerox miljødatablade. Available: www.xerox.dk.

226. Yamamoto T, Yasuhara A, Shiraishi H, Nakasugi O. Bisphenol A in hazardous waste landfill leachates. *Chemosphere* 42:415-418 (2001).

227. Yamamoto, Takashi and Yasuhara, Akio. Quantities of bisphenol a leached from plastic waste samples. *Chemosphere* 38[11]. 1999. Ref Type: Generic

228. Rigol, A., Latorre, A., Lacorte, S., and Barcelo, D. Determination of toxic compounds in paper-recycling process waters by gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. *J Chromat A* 963[1-2] (2002).

229. Fukazawa, Hitoshi, Hoshino, Kentaro, Shiozawa, Tatsushi, Matsushita, Hidetsuru, and Terao, Yoshiyasu. Identification and quantification of chlorinated bisphenol A in wastewater from wastepaper recycling plants. *Chemosphere* 44[5] (2002).

230. Tønning K. Statistik for returpapir og -pap 1999, Miljøprojekt 618. Miljøstyrelsen, Miljø og Energiministeriet, (2001).

231. Uniscrap. Uniscrap homepage. Available: http://www.uniscrap.dk/papir.htm [cited 17 February 2002].

232. Fürhacker, M., Scharf, S., and Weber, H. Bisphenol A: emissions from point sources. *Chemosphere* 41[5] (2000).

233. Vader, J. S., van Ginkel, C. G., Sperling, F. M. G. M., de Jong, J., de Boer, W., de Graaf, J. S., van der Most, M., and Stokman, P. G. W. Degradation of ethinyl estradiol by nitrifying activated sludge. *Chemosphere* 41[8] (2000).

234. Lai, K. M., Johnson, K. L., Scrimshaw, M. D., and Lester, J. N. Binding of Waterborne Steroid Estrogens to Solid Phases in River and Estuarine Systems. Environmental Science and Technology - Columbus 34[18] (2000).

235. Meylan, W. and Howard, P. H. Estimation of log octanol/water partition coefficient. log Kow program. Version 1.35a. Syracuse Research Corporation, Syracuse (1994).

236. Shore LS, Gurevitz M, Shemesh M. Estrogen as an environmental pollutant. *Bull Environ Contam Toxicol* 51:361-366 (1993).

237. Belfroid, A. C., Van der Horst, A., Vethaak, A. D., Schafer, A. J., Rijs, G. B. J., Wegener, J., and Cofino, W. P. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci Tot Environ* 225[1-2]. 1999.

238. Ying, Guang Guo, Williams, Brian, and Kookana, Rai. Environmental fate of alkylphenols and alkylphenol ethoxylates-a review. *Environ Int* 28[3] (2002).

239. Tanghe, Tom and Verstraete, Willy. Nonylphenol degradation in lab scale activated sludge units is temperature dependent. *Water Res* 32[10] (1998).

240. Giger W, Brunner PH, Schaffner C. 4-nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. *Science* 225:623-625 (1984).

241. Corcia, Antonio Di, Samperi, Roberto, and Marcomini, Antonio. Monitoring aromatic surfactants and their biodegradation intermediates in raw and treated sewages by solid-phase extraction and liquid chromatography. *Environ Sci Technol - Columbus* 28[5] (1994).

242. Field, Jennifer A. and Reed, Ralph L. Nonylphenol Polyethoxy Carboxylate Metabolites of Nonionic Surfactants in U.S. Paper Mill Effluents, Municipal Sewage Treatment Plant Effluents, and River Waters. *Environ Sci Technol* - Columbus 30[12] (1996).

243. Corcia, Antonio Di, Costantino, Angela, Crescenzi, Carlo, Marinoni, Erica, and Samperi, Roberto. Characterization of Recalcitrant Intermediates from Biotransformation of the Branched Alkyl Side Chain of Nonylphenol Ethoxylate Surfactants. *Environ Sci Technol* - Columbus 32[16] (1998).

244. Bennie, D. T., Sullivan, C. A., Lee, H. B., and Maguire, R. J. Alkylphenol Polyethoxylate Metabolites in Canadian Sewage Treatment Plant Waste Streams. *Water Qual Res J Can* 33[2] (1998).

245. Pedersen BM, Bøwadt S. Nonylphenol og nonylphenolethoxylater i spildevand og slam. Miljøprojekt 704. Miljøstyrelsen, Miljøministeriet, (2002).

246. Fujita, M., Ike, M., Mori, K., Kaku, H., Sakaguchi, Y., Asano, M., Maki, H., and Nishihara, T. Behaviour of nonylphenol ethoxylates in sewage treatment plants in Japan - biotransformation and ecotoxicity. *Wat Sci Technol* 42[7] (2000). Int Water Assoc.

247. Körner, Wolfgang, Bolz, Ulrike, Sussmuth, Wolfgang, Hiller, Georg, Schuller, Winfried, Hanf, V, and Hagenmaier, Hanspaul. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. *Chemosphere* 40[9-11] (2000).

248. Planas, C., Guadayol, J. M., Droguet, M., Escalas, A., Rivera, J., and Caixach, J. Degradation of polyethoxylated nonylphenols in a sewage treatment plant. Quantitative analysis by isotopic dilution-HRGC/MS. *Water Res* 36[4] (1902).

249. Sheahan, D. A., Brighty, G. C., Daniel, M., Kirby, S. J., Hurst, M. R., Kennedy, J., Morris, S., Routledge, E. J., Sumpter, J. P., and Waldock, M. J. Environmental Chemistry - Estrogenic Activity Measured in a Sewage Treatment Works Treating Industrial Inputs Containing High Concentrations of Alkylphenolic Compounds -- A Case Study. *Environ Toxicol Chem* 21[3] (2002).

250. Matsui S, Okawa Y, Ota R. Experience of 16 years operation and maintenance of the Fukashiba industrial wastewater treatment plant o fthe Kashima petrochemical complex -II Biodegradability of 37 organic substances and 28 process waters. *Water Sci Technol* 20:201-210 (1988).

251. European Comission. Technical guidance documents in support of the comission directive 93/67/EEC on risk assessment for new substances. EU,1996.

252. Fromme H, Küchler T, Otto T, Pilz K, Müller J, Wenzel A. Occurrence of phthalates and bisphenol A and F in the environment. *Water Res* 36:1429-1438 (2002).

253. Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M. Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening. *Environ Sci Technol* - Columbus 32[11]. 1998.

254. Kirk, L. A., Tyler, C. R., Lye, C. M., and Sumpter, J. P. Symposium - Environmental Chemistry - Changes in Estrogenic and Androgenic Activities at Different Stages of Treatment in Wastewater Treatment Works. *Environ Toxicol Chem* 21[5] 2002.

255. Rodgers-Gray, Trevor P., Jobling, Susan, Morris, Steven, Kelly, Carole, Kirby, Sonia, Janbakhsh, Afsaneh, Harries, Jule E., Waldock, Michael J., Sumpter, John P., and Tyler, Charles R. Long-Term Temporal Changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ Sci Technol - Columbus* 34[8] (2000).

256. Boscolo, Boscoletto, Gottardi, F., Milan, L., Pannocchia, P., Tartari, V, Tavan, M., Amadelli, R., Battisti, A. De, Barbieri, A., Patracchini, D., and Battaglin, G. Electrochemical treatment of bisphenol-A containing wastewaters. *J Appl Electrochem* 24[10] (1994).

257. Caza, Nicole and Biswas, N. Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. *Water Res* 33[13] (1999).

258. Blok J, Wösten MAD. Source and environmental fate of natural oestrogens. 22 5085. Association of River Waterworks - RIWA, 2000.

259. Finlay-Moore O, Hartel PG, Cabrera ML. 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. *J Environ Qual* 29:1604-1611 (2000).

260. Nichols DJ, Daniel TC, Moore PA, Edwards DR, Pote DH. Runoff of estrogen hormone 17β-estradiol from poultry litter applied to pasture. *J Environ Qual* 26:1002-1006 (1997).

261. Nichols DJ, Daniel TC, Edwards DR, Moore PA, Pote DH. Use of grass filter strips to reduce 17β-estradiol in runoff from fescue-applied poultry litter. *J Soil Water Conserv* 53:74-77 (1998).

262. Shore LS, Gurevich M, Shemesh M. Estrogen as an environmental pollutant. *Bull Environ Contam Toxicol* 51:361-366 (1993).

263. Ternes TA, Andersen H, Gilberg D, Bornerz M. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. *Anal Chem* 74:3498-3504 (2002).

264. Lee H-B, Peart TE. Determination of 4-nonylphenol in effluent and sludge from sewage treatment plants. *Anal Chem* 67:1976-1980 (1995).

265. Petrovic M, Diaz A, Ventura F, Barcelo D. Simultaneous determination of halogenated derivatives of alkylphenol ethoxylates and their metabolites in sludges, river sediments, and surface, drinking, and wastewaters by liquid chromatography-mass spectrometry. *Anal Chem* 73:5886-5895 (2001).

266. Meesters RJW, Schröder HF. Simultaneous determination of 4-nonylphenol and bisphenol A in sewage sludge. *Anal Chem* 74:3566-3574 (2002).

267. Jensen J, Løkke H. Kemiske stoffer i landbruget. 19. Miljø- og Energiministeriet, Danmarks Miljøundersøgelser:1998.

268. Dizer H, Fischer B, Sepulveda I, Loffredo E, Senesi N, Santana F, Hansen P-D. Estrogenic effect of leachates and soil extracts from lysimeters spiked with sewage sludge and reference endocrine disrupters. *Environ Toxicol* 17:105-112 (2002).

269. Billard R. Spermatogenesis in teleost fish. In: Marshall's physiology of reproduction. 4. ed., Vol. 2 - Reproduction in the male (Lamming G.E., ed).Churchill Livingstone, 1990.

18 Appendix A

Table A.1 Sewage treatment plants (PE: Person Equivalent; COD: Chemical Oxygen Demand; BOD Biological Oxygen Demand; HRT: Hydraulic Retention Time)

Plant & Location	Capacity	Actual loading						Treatment processes	Ref.
			Industrial	COD	BOD	HRT	Daily flow		
	(PE)	(PE)	(%)	(mg/l)	(mg/l)	(h)	(m^3/d)		
Denmark in 1998-2001: Jeksen (Hørning)	140	103	0	440	177		23	Low tech.: Biological sand filter	(Boutrup 2001)
Lyngby (Århus)	100	219	0	602	238		31.1	Low tech.: Biological bio-rotor plant	
Ormslev (Århus)	350	30	0	39	10		83	Low tech.: (root zone plant)	
Tåstrup (Århus)	11	11	0	347	141		2	Low tech.: Mechanical	
Tranbjerg	10,000	8,726	0	~350		21^R	2,207	M, AS, N, D, C, F^R	
Mårslet	3,550	4,166	5	~300		12^R	1,067	M, AS, N, D, C, F^R	
Trige	5,000	2,979	5	~350			619		
Harlev	6,000	1,908	10	~50			1,315		
Marselisborg	220,000	271,676	64	~800				M, AS, N, D, C	
Randers	160,000	103,712	35	480				M, AS, N, D, C	
	160,000	78,578	26	330^R	130^R		$27,000^R$		(Christiansen 2001)
Egå		88,782						M, AS, N, D, C, F	(Boutrup 2001)
		78,959	74				21,427		(Christiansen 2001)
Søholt	105,000	74,273	45	600				M, AS, N, D, C, F	(Boutrup 2001)
Fornæs		51,550						M, AS, N, D, C	
Viby	100,000	45,869	30	600				M, AS, N, D, C, F	
	100,000	$126,000^R$	24	571^R			$28,557^R$		(Christiansen 2001)
Skanderborg		33,069						M, AS, N, C	(Boutrup 2001)
Boeslum		19,660						M, AS, N, D, C	
Beder	$6,000^R$	$4,700^R$	25	354^R		16^R	$1,773^R$	M, AS, N, D, C, F	(Christiansen 2001)
Rønde		3,345	10				1,296	M, AS, N, D, C	
Voel		1,040	0^R		180^R	26^R	433^R	M, AS, N, C	
Balle		636	0				285	M, AS, N,	
Ørum		1,011	0				285	M, AS, N,	

Plant & Location	Capacity	Actual loading						Treatment processes	Ref.
			Industrial	COD	BOD	HRT	Daily flow		
	(PE)	(PE)	(%)	(mg/l)	(mg/l)	(h)	(m^3/d)		
Herning	175,000	110,000	50				32,000	M, C, AS, N, D, P-rem $FeCl_3$, AD	(Pedersen 2002)
Hillerød	60,000	48,700	5				20,400	M, C, AS, N, D, P-rem Al salts Filtration, AD	
Sweden:		3,500	0				Approx. 900	C, AEB	(Larsson 1999)
UK: Southend STW		197,749	Mainly domestic				45,000	PS (April-Oct.), 50 % receives Vitox treatment	(Desbrow 1998)
Harpenden STW		31,200	Mainly domestic				8,250	PF, SF	(Desbrow 1998)
Rye Meads STW		357,000	Mainly domestic				88,500	AS, FS; TL	(Desbrow 1998)
Deephams STW		796,000	Mainly domestic				160,000	AS	(Desbrow 1998)
Naburn STW		388,000	Mainly domestic				20,000	SC, PS, BF, SHS	(Desbrow 1998)
Horsham STW		107,250	Mainly domestic				18,000	BF, SL	(Desbrow 1998)
Billing STW		285,959	Mainly domestic				60,000	EXA	(Desbrow 1998)
Chelmsford STW (1997-1998)		138,000	14						(Rodgers-Gray 2000)
Nederlands: STP A, B & C	300,000-560,000	75-95% of Cap.	Mainly domestic					AS	(Belfroid 1999)
STP D & E	< 100,000	75-95% of Cap.	100					AS	(Belfroid 1999)
Eindh	284,400					11	147,000	AS	(Johnson 2000)
Kral	231,000					18	78,000	AS	(Johnson 2000)
West	296,400					26	39,000	AS	(Johnson 2000)
Germany: Frankfurt/Main (1997)		312,000					41,200	PS, Aerator tank , P rem. by $Fe(II)Cl_2$, FS	(Ternes 1999)

Plant & Location	Capacity	Actual loading						Treatment processes	Ref.
			Industrial	COD	BOD	HRT	Daily flow		
	(PE)	(PE)	(%)	(mg/l)	(mg/l)	(h)	(m^3/d)		
Ulm & New Ulm (1998)		350.000	40					PS, AS, P rem. by $FeCl_3$, FS	(Kuck 2000)
Langenau (2000)		15,000	Mainly domistic					PS, AS, FS	(Kuck 2001)
Blaubeuren (2000)		15,000	Mainly domistic					PS, AS, FS	
Ulm & New Ulm (2000)	350,000	350,000	Domistic & industrial					PS, AS, FS	
Ulm & New Ulm (1998)	350,000	350.000	40				80,000-100,000	PS, AS, N, D, P (biological), FS	(Körner 2000)
Ditzingen (1998)	120,000		50					AS, N, D, P	(Spengler 2001)
Ludwigsburg-Eglosheim (1998)	18,400		20					AS, N, D, P	
Ludwigsburg-Poppenweiler (1998)	31,000		30					AS, P	
Stuttgart-Mühlhausen (1998)	1,000,000		15					AS, N, D, P	
Stuttgart-Möhringen (1998)	149,500		20					AS, N, D, P	
Stuttgart-Büsnau (1998)	10,000		0					AS, N, D, P, Ms	
Hechingen (1998)	57,000		50					AS, N, D, P, E (F)[1]	
Albstadt-Ebingen (1998)	150,000		60					AS, N, D, P, E, F	
Sindelfingen (1998)	226,000		50					T, N, P	
Donaueschingen (1998)	148,000		35					AS, N, D, P	
Blaubeuren (1998)	11,000		10					AS, N, D, P	
Ulm (1998)	350,000		40					AS, N, D, P	
Pforzheim (1999)	250,000		50					AS, N, D, P	

Plant & Location	Capacity	Actual loading						Treatment processes	Ref.
			Industrial	COD	BOD	HRT	Daily flow		
	(PE)	(PE)	(%)	(mg/l)	(mg/l)	(h)	(m^3/d)		
Industrial STP 1 (1999)	67,000		100					AS, N, D, P	
Industrial STP 2 (1999)	100,000		85					AS, T, N	
Lahr (1999)	115,000		40					AS, T, N, P	
Waiblingen (1999)	70,000		40					AS, N, D, P	
Leutkirch (1999)	85,000		70					AS, N, P	
39 different STPs (1997)									(Fromme 2002)
Austria: 1 STP	120,000		60				36,000	M, AS (2 tank in series), N, D, FS, AD (sludge age: 8-9 days)	(Fürhacker 2000)
Switzerland: 1983-85 Nänikon		4,000-240,000	Significant amount from a chem. industry	240[5]	101[5]			PS, AS, FS, AD	(Ahel 1994)
Fällanden				210[5]	77[5]				
Bassersdorf				250[5]	83[5]				
Dübendorf				250[5]	96[5]				
Zürich-Glatt				230[5]	73[5]				
Opfikon				280[5]	143[5]				
Niederglatt				250[5]	101[5]				
Bülach				240[5]	108[5]				
Stadel				180[5]	64[5]				
Glattfelden				270[5]	122[5]				
Rheinsfelden				290[5]	101[5]				
Italy: Ostia, Rome (1991-92)									(Corcia 1994)
Cobis , Rome (1997)			Mainly domestic						(Corcia 1998)

Plant & Location	Capacity	Actual loading						Treatment processes	Ref.
			Industrial	COD	BOD	HRT	Daily flow		
	(PE)	(PE)	(%)	(mg/l)	(mg/l)	(h)	(m^3/d)		
Cobis (1999-2000)		40,000	Mainly domestic	540	270	12	10,000	AS	(Baronti 2000)
Ostia		350,000	Mainly domestic	350	220	14	112,000	AS	
Fregene		120,000	Mainly domestic	330	210	12	42,000	AS	
Roma Nord		800,000	Mainly domestic	220	120	14	354,000	AS	
Roma Sud		$1.2x10^6$	Mainly domestic	200	100	12	734,000	AS	
Roma Est		800,000	Mainly domestic	230	140	14	265,000	AS	
USA: STP 1 California							43,200[3]	PT, AS, MFO; HOCl	(Huang 2001)
STP 2 California							630,720[3]	PT, AS,BNU, EF, HOCl	
STP 3 California							19,008[3]	PT, ST, C^2, MF, RO, O_3/UV	
STP 4 California							5,184[3]	PT, T, UV	
STPs in Texas									(Rudel 1998)
Septic tanks in Texas									
6 STPs at Green Bay (1995)									(Field 1996)
Japan: 27 STPs (1998-99)								PS, AS, FS, disinfection (N, D, P at some plants)	(Nasu 2001)
40 STPs (1995-96)								Main part: PS, AS, FS, disinfection	(Fujita 2000)
Canada: Burlington, Ontario		134,000					80,000	AS, AN	(Bennie 1998)
Burnaby, British Columbia		160,000					82,000	PS, 2-stage sedimentation (sludge)	
Cambridge-Galt, Ontario		70,000					50,000	AS, sand F, AN	

Plant & Location	Capacity	Actual loading						Treatment processes	Ref.
			Industrial	COD	BOD	HRT	Daily flow		
	(PE)	(PE)	(%)	(mg/l)	(mg/l)	(h)	(m^3/d)		
Charlottetown, Prince Edwards Island		17,000					10,000	PS, 2-stage sedimentation (sludge)	
Cowansville, Quebec		12,000					17,000	PS, sedimentation of sludge	
Edmonton, Alberta		630,000					230,000	AS	
Granby, Quebec		39,000					53,000	PS, sedimentation of sludge	
Guelph, Ontario		90,000					42,000	Rotating bio-contacters, sand F, AN	
Hamilton, Ontario		320,000					320,000	AS, AN	
Moncton, New Brunswick		61,000					44,000	PS, sedimentation of sludge	
Montreal, Qebec		1,000,000					890,000	PS, $FeCl_3$ coagulation, sedimentation of sludge	
Regina, Saskatchewan		185,000					75,000	AS, BNU, AN	
Saint John, New Brunswick		65,000					18,000	Rotating bio-contacters, AN	
Toronto, Ontario		600,000					430,000	AS, AN	
Truro, Nova Scotia		13,000					Not reported	PS, sedimentation of sludge	
Winnipeg, Manitoba		620,000					340,000	AS with pure O_2, AN	
Ontario (1997-98)		140,000						PS, AS, FS, Clorination in the summer	(Lee 1998b)
Burligton									(Lee 1998a) (Lee 2000)
Dundas									(Lee 1998a)
Edmonton									(Lee 1998a)
Calgary									(Lee 2000)
Galt									(Lee 2000)
Toronto									(Lee 2000)

Plant & Location	Capacity	Actual loading						Treatment processes	Ref.
			Industrial	COD	BOD	HRT	Daily flow		
	(PE)	(PE)	(%)	(mg/l)	(mg/l)	(h)	(m^3/d)		
Brazilian Penha/rio de Janeiro		624,000					120,096	PS, AS (71 %)[4] & T (29 %)[4],	(Ternes 1999)

PS: Primary sedimentation; PF: Percolating filters; SF: Sand filters; AS: Activated sludge; FS: Final sedimentation; TL: Tertiary lagoons; SC: Screening; BF: Biological filtration; SHS: Secondary humus settlement; SL: Settlement lagoons; EXA: Extended aeration; C: Chemical treatment, AEB: Aerobic biological treatment; HRT: Hydraulic retention time; P: Phosphate elimination; N: Nitrification, D: Denitrification; E: Decolorization; Ms: Micro sieve; T: Trickling filter; F: Filtration; MF: Micro filtration; BNU: Biological nutrient removal; EF: Effluent filtration; ST: Secondary treatment but not specified; UV: Ultra violet; AN: Anaerobic digester

R: Information received from the STP
1: At he second time of sampling, this STP was equipped with an additional activated charcoal filtration.
2: Alum flocculation
3: Design flow
4: % of the flow rate
5: COD and BOD in the primary effluent

Table A.2 Concentrations of estrogens in STPs

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
Denmark: Jeksen	Estrone					4.1		(n=3)	(Christiansen 2001)
	17β-estradiol					<0.4			
	17α-ethinylestradiol					<0.5			
Lyngby	Estrone					2.8			
	17β-estradiol					n.a.			
	17α-ethinylestradiol					n.a.			
Ormslev	Estrone					1.3; 108			
	17β-estradiol					<0.4; 31			
	17α-ethinylestradiol					0.2; <1			
Tåstrup	Estrone					27			
	17β-estradiol					n.a.			
	17α-ethinylestradiol					n.a.			
Randers	Estrone					0.5-1.2			
	17β-estradiol					<1			
	17α-ethinylestradiol					<1			
Egå	Estrone					0.3-1.2			
	17β-estradiol					<1			
	17α-ethinylestradiol					<1			
Viby	Estrone					1.2-2.0			
	17β-estradiol					<1			
	17α-ethinylestradiol					<1			
Beder	Estrone					0.5-1.1			
	17β-estradiol					<1-0.6			
	17α-ethinylestradiol					<1-1.4			
Rønde	Estrone					0.3-1.8			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
	17β-estradiol					<1-1.7			
	17α-ethinylestradiol					<1			
Voel	Estrone					<1-6.1			
	17β-estradiol					<1			
	17α-ethinylestradiol					<1			
Balle	Estrone					0.4; 2.5			
	17β-estradiol					<1			
	17α-ethinylestradiol					<1			
Ørum	Estrone					0.3-2.7			
	17β-estradiol					<1-2.5			
	17α-ethinylestradiol					<1-4.7			
Sweden:	Estrone					~6		Continuously sampling in 72-h (n=1)	(Larsson 1999)
	17β-estradiol					~1			
	17α-ethinylestradiol					~4			
	Glucuronider					<0.5			
UK: Southend STW	Estrone					31-49		Analyses were only performed in sample fractions with estrogenic activity. The concentrations may, therefore, be under estimated. Discrete samples (n=3).	(Desbrow 1998)
	17β-estradiol					28-54			
	17α-ethinylestradiol					Nd-7.4			
Harpenden STW	Estrone					4.6-9.7			(Desbrow 1998)
	17β-estradiol					3.1-8.1			
	17α-ethinylestradiol					Nd			
Rye Meads STW	Estrone					1.3-4.0			(Desbrow 1998)
	17β-estradiol					2.6-6.5			
	17α-ethinylestradiol					Nd			
Deephams STW	Estrone					2.0-18			(Desbrow 1998)
	17β-estradiol					3.8-15			
	17α-ethinylestradiol					Nd			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
Naburn STW	Estrone					14-86			(Desbrow 1998)
	17β-estradiol					5.3-12			
	17α-ethinylestradiol					0.4-4.8			
Horsham STW	Estrone					5.5-12.5			(Desbrow 1998)
	17β-estradiol					3.6-6.4			
	17α-ethinylestradiol					0.1-0.9			
Billing STW	Estrone					1.3-11			(Desbrow 1998)
	17β-estradiol					6.1-8.0			
	17α-ethinylestradiol					Nd			
Chelmsford STW (1997-1998)	Estrone					15-220 (A) 27-56 (B)		5 days composite samples; Taken at 12 pm daily. (n=4 in each of two trials A and B)	(Rodgers-Gray 2000)
	17β-estradiol					7-88 (A) 4-8.8 (B)			
Nederlands: STP A, B & C	Estrone					<0.4-47		Samples were collected over a 7-h period. (n=2 from each plant)	Belfroid (1999)
	17β-estradiol					<0.6-12			
	17α-estradiol					<0.1-5.0			
	17α-ethinylestradiol					<0.2-7.5			
	Hormone glucuconides					<1.4- 2.7[1]			
STP D & E	Estrone					<0.1-11		Samples were collected over a 7-h period. (n=2 from each plant)	Belfroid (1999)
	17β-estradiol					<0.4-1.8			
	17α-estradiol					<0.1-2.1			
	17α-ethinylestradiol					<0.2-2.6			
	Hormone glucuconides					<1.4-7.4[1]			
Eindh	Estrone	11; 42				2.7; 15	64; 75	Composite of samples collected every 30 min's kl. 7-15. (n=2)	(Johnson 2000)
	17β-estradiol	11; 14				1.1	Na; 92		
	Estriol	No				No	Na		
	17α-ethinylestradiol	<0.5; <1.4				<0.5; <1.4	Na		
Kral	Estrone	18; 100				<0.4; 6.3	94; >98		

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
	17β-estradiol	31				0.7	98		
	Estriol	No				No			
	17α-ethinylestradiol	<0.2; <1.4				<0.2; <1.8	Na		
West	estrone	87; 140				2.1; 47	66; 98		
	17β-estradiol	9; 48				<0.6, 12	75; >94		
	estriol	No				No			
	17α-ethinylestradiol	1.3; 8.8				<0.2; <0.3	77; >98		
Germany: Frankfurt/Main (1997)	Estrone	27	>50			23		Flow proportional sampling. 6 days composite samples (n=1)	(Ternes 1999)
	17β-estradiol	15	19			5.4	64		
	16α-hydroxyestrone	13	15			4.4	68		
	17α-ethinylestradiol	1.2	1.5			2.7	~ 0		
Ulm & New Ulm (1998)	Estrone					3-13		6-h sampling period (n=7)	(Kuch 2000)
	17β-estradiol					1-13			
	Estriol					<1-9			
	17α-ethinylestradiol					<1-5			
Ulm & Neu-Ulm, Langeneau and Blaubeuren (2000)	Estrone					0.35-18		Grab samples taken in the morning (n=16)	(Kuch 2001)
	17β-estradiol					0.15-5.2			
	17α-estradiol					0.15-4.5			
	17α-ethinylestradiol					0.1-8.9			
Ditzingen (1998)	Estrone					17		24-h time-proportional sampling (n=1 from each plant)	(Spengler 2001)
	17β-estradiol					4.2			
	17α-ethinylestradiol					<1.2			
Ludwigsburg-Eglosheim (1998)	Estrone					<1.8			
	17β-estradiol					<0.4			
	17α-ethinylestradiol					<1.2			
Ludwigsburg-Poppenweiler (1998)	Estrone					<0.7			
	17β-estradiol					<1.2			
	17α-ethinylestradiol					<1.2			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
Stuttgart-Mühlhausen (1998)	Estrone					<0.7			
	17β-estradiol					2.6			
	17α-ethinylestradiol					<1.2			
Stutgart-Möhringen (1998)	Estrone					3.1			
	17β-estradiol					6.4			
	17α-ethinylestradiol					4.1			
Stuttgart-Büsnau (1998)	Estrone					7.5			
	17β-estradiol					1.6			
	17α-ethinylestradiol					2.7			
Hechingen (1998 & 1999))	Estrone					22			
	17β-estradiol					15			
	17α-ethinylestradiol					No result			
Alstadt-Ebingen (1998)	Estrone					<1.8			
	17β-estradiol					<1.2			
	17α-ethinylestradiol					<1.2			
Sindelfingen (1998)	Estrone					18			
	17β-estradiol					5.4			
	17α-ethinylestradiol					12			
Donaueschingen (1998)	Estrone					3.5			
	17β-estradiol					4.4			
	17α-ethinylestradiol					<0.4			
Blaubeuren (1998)	Estrone					4.3			
	17β-estradiol					2.9			
	17α-ethinylestradiol					2.4			
Ulm (1998)	Estrone					5.1			
	17β-estradiol					<0.4			
	17α-ethinylestradiol					2.0			
Pforzheim	Estrone					0.8			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
(1999)	17β-estradiol					1.1			
	17α-ethinylestradiol					2.3			
Industrial STP 1 (1999)	Estrone					<0.7			
	17β-estradiol					<0.4			
	17α-ethinylestradiol					<0.4			
Industrial STP 2 (1999)	Estrone					<0.7			
	17β-estradiol					7.1			
	17α-ethinylestradiol					No result			
Lahr (1999)	Estrone					10			
	17β-estradiol					0.8			
	17α-ethinylestradiol					2.0			
Waiblingen (1999)	Estrone					1.4			
	17β-estradiol					1.7			
	17α-ethinylestradiol					0.4			
Leutkirch (1999)	Estrone					0.2			
	17β-estradiol					<1.2			
	17α-ethinylestradiol					0.9			
Israel	Estrogenes	48-141				7-50	20-88		(Shore 1993)
Italy: Cobis[2]	Estrone	42-132				5.4-17	E1: 61 ± 38 % E2: 87 ± 9 % E3: 95 ± 6 % EE2: 85 ± 14 %	24-h flow proportional composite samples. One per month over 5 months (n=5)	(Baronti 2000)
	Estradiol	8.1-25				0.55-2.9			
	Estriol	44-188				1.1-7.3			
	Ethinylestradiol	0.45-13				Nd-1.0			
Ostia[2]	Estrone	33-67				13-82.1			
	17β-estradiol	5.9-22				0.72-3.5			
	Estriol	54-187				0.63-1.5			
	17α-ethinylestradiol	0.52-4.8				Nd-1.1			
Fregene[2]	Estrone	41-87				2.5-6.5			
	Estradiol	4.0-16				0.35-2.1			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
	Estriol	26-127				0.44-2.2			
	Ethinylestradiol	0.44-6.8				Nd-1.7			
Roma Nord[2]	Estrone	30-49				6.4-40			
	Estradiol	6.3-14				0.44-1.9			
	Estriol	35-148				0.72-8.4			
	Ethinylestradiol	0.46-6.8				Nd-0.56			
Roma Sud[2]	Estrone	25-48				8.7-51			
	Estradiol	4.7-10				0.53-3.1			
	Estriol	24-127				1.8-18			
	Ethinylestradiol	0.43-6.1				Nd-1.2			
Roma Est[2]	Estrone	34-68				3.7-10			
	Estradiol	6.3-11				0.62-0.82			
	Estriol	33-146				0.43-1.4			
	Ethinylestradiol	0.40-4.6				Nd-0.73			
USA, California STP1	17β-estradiol					2.75-4.05		Analyzed with ELISA (n=1-4)	(Huang 2001)
	17α-ethinylestradiol					1.54-2.42			
California STP2	17β-estradiol					0.38-1.01			
	17α-ethinylestradiol					0.19-0.49			
California STP3	17β-estradiol			0.72-2.65[5] <0.1-0.32[6]					
	17α-ethinylestradiol			0.13-0.16[5] <0.1[6]					
California STP 4	17β-estradiol					0.2			
	17α-ethinylestradiol					0.66			
Japan: 27 STPs (1998-1999)	17β-estradiol	20-94				0.2-55	(7->99 in the winter)	Range for 27 STPs	(Nasu 2001)
		45 (58[3])	66	20		20[4]		Example of the fate	
Canada:	Estrone		26; 53			6; 8		Grab or 24-h composite	(Lee 1998a)

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge	Final effluent	Rem/loss	Remarks	Ref.
		Conc. (ng/l)					%		
Burlington	17β-estradiol		7; 14			<5; <5		samples (n= 1-3)	
	Estriol		128; 220			18; 33			
Dundas	Estrone		68, 70			8, 10			
	17β-estradiol		8, 9			<5, <5			
	Estriol		203; 243			<10; <10			
Edmonton	Estrone		109			72			
	17β-estradiol		<5			<5			
	Estriol		209			<10			
Guelph	Estrone	41-75				14-18			
	17β-estradiol	<5; 15				<5; <5			
	Estriol	158-250				30-37			
Brazilian Penha/Rio de Janeiro	Estrone	40				14 (T); 6.8 (AS)	67 (T); 83 (AS)	Random samples taken over 6 days and combined (n=1)	(Ternes 1999)
	17β-estradiol	21				1.7 (T); 0.2 (AS)	92 (T); 99.9 (AS)		
	Ethinylestradiol	6				2 (T); 1.3 (AS)	64 (T); 78 (AS)		

A: Trial from November to March with temperature at 12.3 ± 0.4°C
B: Trial from July to December with temperature at 17.2 ± 0.7°C
Nd: Not detected, nq: below the limit of quantification; no: not determined; na: not applicable
T: Trickling filter
AS: Activated sludge
1: Under detection level except for 1-2 sample (E1 = estron)
2: 24 h flow proportional sampling
3: Influent into primary settling tank
4: After disinfection
5: After microfiltration
6: After reverse osmosis

Table A.3 Concentrations of alkylphenoles and parent compounds

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
Danmark: Jeksen	NpnEO, n = 3-15	<20-36				<20	70	(n=6)	(Boutrup 2001)
	NP+NPnEO, n = 1-2	3.6-9.8				0.23-1.02	92		
	OpnEO, n = 3-15	<20-30				<20	67		
	OpnEO, n = 1-2								
	OP	<0.1-<1				<0.1	79		
Lyngby	NPnEO, n = 3-15	<20-51				<20	74	(n=6)	
	NP+NPnEO, n = 1-2	2.8-8.7				0.36-2.13	75		
	OPnEO, n = 3-15	<20				<20			
	OPnEO, n = 1-2								
	OP	<0.1-<1				<0.1-<0.4			
Ormslev	NPnEO, n = 3-15	<20				<20		(n=6)	
	NP+NPnEO, n = 1-2	<0.4-2.26				0.10-0.28	82		
	OPnEO, n = 3-15	<20				<20			
	OPnEO, n = 1-2								
	OP	<0.1				<0.1			
Tåstrup	NPnEO, n = 3-15	<20				<20	-6	(n=6)	
	NP+NPnEO, n = 1-2	2.0-23				1.83-7.62	18		
	OPnEO, n = 3-15	<20				<20			
	OPnEO, n = 1-2								
	OP	<0.1-<0.5				<0.1-<0.5			
Marselisborg	NPnEO, n = 3-15	24-39				<20		(n=4-5)	
	NP+NPnEO, n = 1-2	5.1-9.8				0.15-0.38			
	OPnEO, n = 3-15	<20				<20			
	OPnEO, n = 1-2					n.a.			
	OP	<0.1				<0.2			
Randers	NPnEO, n = 3-15	<20-40				<20		(n=4-5)	

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	NP+NPnEO, n = 1-2	8.6-18				0.24-1.2			
	OPnEO, n = 3-15	<20				<20			
	OPnEO, n = 1-2					n.a.			
	OP	<0.1				<0.2			
Egå	NPnEO, n = 3-15					<20		(n=1)	
	NP+NPnEO, n = 1-2					<0.6			
	OPnEO, n = 3-15					<20			
	OPnEO, n = 1-2					n.a.			
	OP					n.a.			
Søholt	NPnEO, n = 3-15							(n=3)	
	NP+NPnEO, n = 1-2	36-96				1.6-7.4			
	OPnEO, n = 3-15	<20				n.a.			
	OPnEO, n = 1-2					n.a.			
	OP	<0.1				<0.3			
Fornæs	NPnEO, n = 3-15					<20		(n=1)	
	NP+NPnEO, n = 1-2					0.57			
	OPnEO, n = 3-15					<20			
	OPnEO, n = 1-2					n.a.			
	OP					<0.3			
Viby	NPnEO, n = 3-15	n.a.						(n= 3)	
	NP+ NPnEO, n = 1-2	43-52				0.34-0.61			
	OPnEO, n = 3-15	<20				n.a.			
	OPnEO, n = 1-2					n.a.			
	OP	<0.1				n.a.			
Skanderborg	NpnEO, n = 3-15					<20		(n=1)	
	NP + NPnEO, n = 1-2					<0.6			
	OpnEO, n = 3-15					<20			
	OpnEO, n = 1-2					n.a.			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	OP					<0.1			
Boeslum	NpnEO, n = 3-15					<20		(n=1)	
	NP + NPnEO, n = 1-2					0.35			
	OpnEO, n = 3-15					n.a.			
	OpnEO, n = 1-2					n.a.			
	OP					<0.2			
Herning (1998-99)	NpnEO, n = 3-18	25.1-40	31.1; 32.1	2.42; 2.82[1]	<0.1; <0.1[2]				(Pedersen 2002)
	OpnEO; n = 3-18	<1-<20	<1; <1	<1; <11[1]	<0.1; <0.1[2]				
	NP+NPnEO, n = 1-2	0.64-29	5.3; 20	9.1; 17[1]	213; 216[2]				
Hillerød (1998-99)	NpnEO; n = 3-18	3.75-<20			1.13; 1.46[2]				
	OpnEO; n = 3-18	<1-<20			<0.1; <0.1[2]				
	NP+NPnEO, n = 1-2	<0.1-24			41; 46[2]				
Germany: Ulm & New Ulm (1998)	Sum 4-nonylphenol	2.13; 2.59				0.32; 1.57	85; 40	24-h sampling periode (n=2)	(Körner 2000)
	4-t-octylphenol	0.321; 0.183				0.281; 0.357	13; -96;		
Switzerland: 1983-85 Nänikon	NPnEO, n = 3-20		(1480)			(210)	(86)	24- and 2-h composite water samples	(Ahel 1994)
	NP1EO+NP1EO		(510)			(490)	(4)		
	NP1EC+NP1EC		(150)			(610)	(-310)		
	NP		(220)			(200)	(9)		
	SUM NP-c		(2360) 1120			(1510) 430	(36)		
Fällanden	NPnEO, n = 3-20		(1980)			(140)	(93)		
	NP1EO+NP1EO		(310)			(70)	(77)		
	NP1EC+NP1EC		(180)			(600)	(-230)		
	NP		(170)			(10)	(94)		
	SUM NP-c		(2640) 1390			(820) 300	(69)		
Bassersdorf	NPnEO, n = 3-20		(3220)			(110)	(97)		

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	NP1EO+NP1EO		(480)			(10)	(77)		
	NP1EC+NP1EC		(170)			(580)	(-240)		
	NP		(110)			(30)	(73)		
	SUM NP-c		(3980) 2060			(830) 270	(79)		
Dübendorf	NPnEO, n = 3-20		(1570)			(380)	(76)		
	NP1EO+NP1EO		(660)			(470)	(29)		
	NP1EC+NP1EC		(160)			(930)	(-480)		
	NP		(160)			(100)	(37)		
	SUM NP-c		(2550) 1120			(1880) 460	(26)		
Zürich-Glatt	NPnEO, n = 3-20		(1690)			(140)	(92)		
	NP1EO+NP1EO		(450)			(90)	(80)		
	NP1EC+NP1EC		(140)			(440)	(-210)		
	NP		(140)			(20)	(86)		
	SUM NP-c		(2420) 1200			(690) 240	(71)		
Opfikon	NPnEO, n = 3-20		(2980)			(640)	(78)		
	NP1EO+NP1EO		(640)			(760)	(-19)		
	NP1EC+NP1EC		(170)			(590)	(-250)		
	NP		(260)			(160)	(38)		
	SUM NP-c		(4050) 2020			(2150) 760	(47)		
Niederglatt	NpnEO, n = 3-20		(1890)			(160)	(92)		
	NP1EO+NP1EO		(790)			(170)	(78)		
	NP1EC+NP1EC		(90)			(290)	(-220)		
	NP		(270)			(40)	(85)		

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	SUM NP-c		(3040) 1440			(660) 240	(78)		
Bülach	NPnEO, n = 3-20		(2390)			(280)	(88)		
	NP1EO+NP1EO		(530)			(270)	(49)		
	NP1EC+NP1EC		(130)			(490)	(-280)		
	NP		(170)			(30)	(82)		
	SUM NP-c		(3220) 1640			(1070) 370	(67)		
Stadel	NPnEO, n = 3-20		(1310)			(280)	(79)		
	NP1EO+NP1EO		(830)			(620)	(25)		
	NP1EC+NP1EC		(100)			(580)	(-480)		
	NP		(4309)			(70)	(84)		
	SUM NP-c NP-c		(2670) 1090			(1550) 530	(42)		
Glattfelden	NPnEO, n = 3-20		(1730)			(130)	(92)		
	NP1EO+NP1EO		(390)			(140)	(64)		
	NP1EOC+NP1OEC		(80)			(610)	(-660)		
	NP		(130)			(60)	(54)		
	SUM NP-c		(2330) 1220			(940) 300	(60)		
Rheinsfelden	NpnEO, n = 3-20		(2170)			(130)	(94)		
	NP1EO+NP1EO		(840)			(200)	(76)		
	NP1EOC+NP1EOC		(270)			(560)	(-110)		
	NP		(110)			(30)	(73)		
	SUM NP-c		(3390) 1530			(920) 300	(73)		
Italy: Ostia (1991-92)	NPnEO, n = 1-18	50-360				2-27		24-h composite samples collected monthly during	(Corcia 1994)
	NPnEOC, n= 1->7	<~3				10-145			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	NP	2-40				0.7-4		one year (n=12)	
Cobis, Rome (1997)	CAmPEnC, m=3-8, n=1-2					58		A 24-h flow proportional composite sample (n=1)	Corcia 1998)
	CAmPEn, m=6-8, n=1-2					0.68			
	Total A_9PE's (A_9PE_2)					12			
	Total A_9PEC's (A_9PE_2C)					21			
USA: STPs in Texas	NP/OP6EO	81[3]				9.4-86		Grab samples (n= 3-4)	(Rudel 1998)
	NP/OP5EO	2000[3]				12[3]			
	NP/OP4EO					18[3]			
	NP/OP3EO	890-1000							
	NP2EO	6.4-8.0				0.80[3]			
	NP1EO	15-21				5.5[3]			
	NP1EC	1.3-1.7				42[3]			
	NP	25-33				16[3]			
	OP2EO	0.067[3]							
	OP1EO	0.21[3]							
	OP	0.20-0.74				0.15[3]			
Septic tanks in Texas	NP/OP6EO	58[3]						Grab samples (n=5)	
	NP/OP5EO	900[3]							
	NP/OP4EO	77[3]							
	NP/OP3EO	2300[3]							
	NP2EO	79-100							
	NP1EO	440-580							
	NP1EC	37-57							
	NP	1000-1500							
	OP2EO								

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	OP1EO	8.0-9.8							
	OP	35-42							
6 STPs at Green Bay (1995)	NP1EC					7.6-29.4		24-h composite samples (n=6)	(Field 1996)
	NP2EC					64.1-128.0			
	NP3EC					24.8-105.0			
	NP4EC					9.7-29.2			
	SUM NPnEC, n = 1-4					142.5-272.4			
Japan: 40 STPs (1995-96)	NPnEO, n = 4-18		5.1-1035	n.d.-409		n.d.-245		Grab samples (n=38-40)	(Fujita 2000)
	NPnEO, n = 1-3		n.d.-938	n.d.-42.9		n.d.-60.0			
	NPnEC, n = 1-3		n.d.-25.8	n.d.-22.9		n.d.-1119			
	BrNPnEO, n = 1-2			n.d.-0.5		n.d.-0.1			
	BrNPnEC, n = 1-2			n.d-0.9		n.d.-52.4			
	ClNPnEO, n = 1-2			n.d.-6.5		n.d.-4.0			
	ClNPnEC, n = 1-2			n.d.-1.4		n.d.-3.3			
	NP			n.d.-3.9		n.d.-1.7			
	SUM NP-c		10.3-1972	0.2-454		n.d.-1171	Average = 70		
Canada: Ontario	NPnEO, n = 1-17	(275-671) 123-415	(109-342) 43.1-167			(11.4-96.7) 3.3-32.4		24-h composite samples (n=12)	(Lee 1998b)
	NP	(8.2-103)	(7.2-49.6)			(2.6-9.6)			
	NP1EC	(7.9-257)	(11.9-39.9)			(32.7-158)			
	NP2EC	(4.4-47.8)	(13.4-50.9)			(41-128)			
	OP	(0.8-7.5)	(0.6-3.9)			(0.2-3.2)			
	OP1EC	(0.8-31.8)	(0.9-11.5)			(4.9-109)			
	OP2EC	(0.3-12.4)	(1.1-10.2)			(4.9-41)			
	Total NP-c+OP-c	(319-844)	(155-469)			(169-484)	(20-76)		
Burlington, Ontario	NP2EO	0.88			2.6	0.16		Grab samples	(Bernie 1998)
	NP1EO	3.6			29	0.15			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	4-NP	28			229	0.60			
	4-t-OP	1.8			5.9	0.030			
Burnaby, British Columbia	NP2EO				26	2.4			
	NP1EO				54	5.3			
	4-NP				469	13			
	4-t-OP				9.7	<0.005			
Cambridge-Galt, Ontario	NP2EO	1.3			42	12			
	NP1EO	3.2			167	26			
	4-NP	156			329	1.4			
	4-t-OP	21			15	<0.005			
Charlottetown, Prince Edwards Island	NP2EO				20	1.7			
	NP1EO				71	3.3			
	4-NP				45	2.5			
	4-t-OP				3.0	0.37			
Cowansville, Quebec	NP2EO	24			43	2.1			
	NP1EO	43			26	1.5			
	4-NP	62			11	0.37			
	4-t-OP	<0.005			<0.010	<0.005			
Edmonton, Alberta	NP2EO				297	4.8			
	NP1EO				332	6.9			
	4-NP				159	0.55			
	4-t-OP				5.3	<0.005			
Granby, Quebec	NP2EO	6.5			69	0.67			
	NP1EO	4.3			14	0.072			
	4-NP	2.3			8.4	0.62			
	4-t-OP	0.12			1.1	0.054			
Guelph, Ontario	NP2EO	0.26			23	21			
	NP1EO	5.3			36	14			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	4-NP	119			850	1.9			
	4-t-OP	6.3			20	<0.005			
Hamilton, Ontario	NP2EO	0.59			1.5	0.54			
	NP1EO	5.9			25	1.3			
	4-NP	12			609	1.6			
	4-t-OP	1.2			16	0.18			
Moncton, New Brunswick	NP2EO	4.3			14	4.4			
	NP1EO	2.9			70	3.0			
	4-NP	0.69			15	1.8			
	4-t-OP	0.14			0.70	0.13			
Montreal, Qebec	NP2EO				34	1.7			
	NP1EO				128	2.5			
	4-NP				113	1.2			
	4-t-OP				5.3	0.12			
Regina, Saskatchewan	NP2EO				93	0.46			
	NP1EO				437	1.11			
	4-NP				198	<0.020			
	4-t-OP				8.1	<0.005			
Saint John, New Brunswick	NP2EO				20	0.38			
	NP1EO				3.9	0.26			
	4-NP				11	0.45			
	4-t-OP				0.87	0.049			
Toronto, Ontario	NP2EO	n.a.			26	0.099			
	NP1EO	n.a.			70	0.092			
	4-NP	5.5			272	1.7			
	4-t-OP	0.56			7.9	0.060			
Truro, Nova Scotia	NP2EO	8.4			93	5.4			
	NP1EO	8.5			70	1.5			

Plant & Location	Compound	Influent	Effluent after PS	Effluent after FS	Sludge (Conc. in µg/g dry weight)	Final effluent	Rem/loss	Remarks	Ref.
		Conc. in water samples in µg/l (values in brackets are in µmol/m^3)					%		
	4-NP	2.9			37	1.8			
	4-t-OP	0.28			0.52	0.18			
Winnipeg, Manitoba	NP2EO				34	1.8			
	NP1EO				62	1.0			
	4-NP				239	4.8			
	4-t-OP				6.7	<0.005			

n.d.: not detectable
n.a.: not aviable
1: Reject water
2: De-watered sludge (dry matter = 24-26%)
3: Average of detected

Table A.4 Concentrations of bisphenol A in STPs

Plant & Location	Influent	Sludge	Final effluent	Rem/loss	Remarks	Ref.
	Conc. in samples of water (ng/l)			%		
Denmark in 1998-2001: Jeksen (Hørning)	410-1,100		50-920	92-96	(n=6)	(Boutrup 2001)
Lyngby (Århus)	<500-1,300		<100-1,100	40-72	(n=3-6)	
Ormslev (Århus)	<500-600		<100-130	41-92	(n=3)	
Tåstrup (Århus)	<100-2,600		380–1,800	-20-38	(n=5-6)	
Tranbjerg	560				(n=1)	
Mårslet	<100				(n=1)	
Trige	<100				(n=1)	
Harlev	1,500				(n=1)	
Marselisborg	<100-1,300		<500-1,400		(n=5)	
Randers	700-3,000		<500-1,200		(n=5)	
			<100-4,000		(n=3)	(Christiansen 2001)
Egå			<500		(n=1)	(Boutrup 2001)
			<100-<100		(n=3)	(Christiansen 2001)
Søholt			<500-<500		(n=3)	(Boutrup 2001)
Fornæs			<500		(n=1)	(Boutrup 2001)
Viby	3,400-17,000		<500-(160?)		(n=3)	(Boutrup 2001)
			<100-<100		(n=3)	(Christiansen 2001)
Skanderborg			<500		(n=1)	(Boutrup 2001)
Boeslum			<500		(n=1)	
Beder			<100-<100		(n=3)	(Christiansen 2001)
Rønde			<100-<100		(n=3)	
Voel			<100-2,200		(n=3)	
Balle			<100-160		(n=3)	
Ørum			<100-<100		(n=3)	

Plant & Location	Influent	Sludge	Final effluent	Rem/loss	Remarks	Ref.
	Conc. in samples of water (ng/l)			%		
Sweden			~500		Continuously sampling in 72-h (n=1)	(Larson 1999)
Germany: Ulm & Neu-Ulm (1998)	542; 3,010		162; 258	70; 91	24-h sampling period (n=2)	(Körner 2000)
Ulm & Neu-Ulm, Langenau, Blaubeuren (2000)			4.8-47		Grab samples taken in the morning (n=16)	(Kuch 2001)
Ditzingen (1998)			130		24-h time-proportional sampling (n=1 from each STP)	(Spengler 2001)
Ludwigsburg-Eglosheim (1998)			40			
Ludwigsburg-Poppenweiler (1998)			40			
Stuttgart-Mühlhausen (1998)			100			
Stuttgart-Möhringen (1998)			80			
Stuttgart-Büsnau (1998)			50			
Hechingen (1998)			80			
Albstadt-Ebingen (1998)			30			
Sindelfingen (1998)			1,000			