ASSESSMENT OF ENVIRONMENTAL ESTROGENS IN WASTEWATER: POTENTIAL FOR DEVELOPMENTAL AND REPRODUCTIVE TOXICITY IN FISH

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INTRODUCTION

Anthropogenic chemicals may adversely affect populations via several mechanisms. Far-reaching consequences can occur at the organismal and population levels when there are interferences of biological systems at the molecular level. Chemicals that disrupt the endocrine system have negative results on the population by virtue of developmental and reproductive impairment. One class of these endocrine disruptors are compounds that mimic or antagonize the effect of endogenous estrogens, referred to as environmental estrogens or xenoestrogens. A number of environmental contaminants possess estrogenic activity including degradation products of high-use industrial surfactants and by-products of industrial processes such as pulp and paper mill production. Compounds such as alkyl phenol ethoxylates (APEs) and nonylphenol, a break down product of APEs, have been shown to possess estrogenic activity (Ren et al. 1996; Routledge and Sumpter 1996; White et al. 1994; Jobling et al. 1995, 1996), as have several other environmentally-relevant chemicals. Polychlorinated biphenyls (PCBs), the pesticides DDT, methoxychlor and chlordecone, even natural plant and fungal products have been shown to be estrogenic. Also included in this list of environmental estrogens are potent natural and synthetic estrogens which find their way to the aquatic environment through animal waste or municipal sewage effluent (Figure 1).

In a recent investigation released by the U.K. Environment Agency, it was reported that the synthetic estrogen, ethinyl estradiol, and endogenous estradiol and estrone, significantly contributed to the estrogenic activity of select sewage effluents (U.K. Environment Agency 1996). These estrogenic compounds are used extensively in oral contraceptive formulations, hormone replacement therapy, and as growth enhancement agents in livestock production. In addition, natural estradiol and estrone are excreted daily by women of reproductive age and female animal livestock. Concerns regarding potential environmental exposure to pharmaceutical estrogens arise because of their greater potency in relation to the well studied environmental estrogens. For example, APEs and some organochlorine compounds are relatively weak estrogens, having 1/50th to 1/10, 000 the potency of estradiol (Arnold 1996). However, there is little information regarding the environmental fate of pharmaceuticals because it is usually assumed that environmental concentrations are lower than those required for biological effects.

Most environmental estrogens act by a similar mechanism as estradiol. Estradiol induces its biological effects by binding to a protein, the estrogen receptor, and this steroid:recetor complex interacts with DNA to promote protein synthesis. However, most environmental estrogens have less affinity for the estrogen receptor, thus, less potency than E2. There are also some recognized indirect mechanisms of estrogenic activity such as interference with enzymes in the steroidal metabolic pathways or interaction at points along the reproductive axis upstream of the target tissue (e.g., hypothalamus or pituitary). The survival of a species depends on successful development and reproduction. Therefore, considering the role of sex steroids in the regulation of reproductive processes and development of reproductively competent organisms, environmental estrogens have the potential to be devastating to populations.

Environmental estrogens may compete with the effect of endogenous estradiol either by binding at the receptor or through negative feedback pathways in the hypothalamic-pituitary-gonadal axis. As such, environmental estrogens have the potential to perturb delicate hormone pathways which may result in decreased fertility and egg production. Also, because the germ cells of developing fish are bipotential and the inducers of gonadal development are most likely the sex steroid hormones, estrogenic compounds have the potential to skew the gender ratio to female. Genetic males which have been phenotypically reversed to female may not have the same reproductive competence as their genetic female counterparts. Reproductive toxicity may occur during larval development, or, because many of these compounds are designed to be resistant to degradation and metabolism, they can be bioaccumulated and may compromise the successful maturation of the gonads in a later stage. The reproductive role of adult female fish is to produce viable eggs and offspring. The process of producing such eggs is dependent upon hormonal communication between the brain, ovary, and liver. Estrogen receptors present in the involved organs coordinate the delicate timing of the vitellogenesis/maturing/ovulating sequence. If

something goes awry, eggs are not spawned or developing embryos do not have an adequate nutrition source for development.

The following is a review of the literature that describes the background material which initiated our current research activities sponsored by the Mississippi Water Resources Research Institute. This manuscript describes the basis for the hypothesis that pharmaceutical products are reaching aquatic systems at biologically active concentrations. A brief overview of the current uses of estrogen in the United States is presented. From these data an estimate of the concentration of natural and synthetic estrogens reaching wastewater receiving stations is calculated using an expected introduction concentration. Finally, the biochemical processes that alter these compounds occurring in humans and microbial populations in wastewater treatment plants is also reviewed.

ESTROGEN USE

Estrogenic pharmaceutical products are used in both human and veterinary medicine. As oral contraceptives and hormone replacement therapy, millions of women take estrogen each day. Likewise, millions of feedlot cattle are administered estrogens for growth enhancement purposes. The following discussion focuses on the major human and veterinary uses of estrogenic pharmaceutical products. Table 1 outlines the human uses of estrogenic pharmaceutical products.

Oral Contraceptives

In 1990, there were over 58 million American women between the ages of 15 to 44 that practiced contraception. Of the 58 million women, approximately 10 million used oral contraceptives (Peterson 1995). Oral contraceptives typically contain a combination of estrogen and progestin, with the active ingredients being ethinyl estradiol and a 19-nor progesterone (Williams and Stancel 1996). Mestranol is sometimes utilized instead of ethinyl estradiol, but is administered at a higher dose due to lower potency. The concentration of ethinyl estradiol in the contraceptive pill ranges from 20 to 50 ug, with 35 ug most commonly prescribed. The regimen consists of taking the "pill" for 21 consecutive days, followed by 7 days without intake, prior to the next 21 day cycle.

Hormone Replacement Therapy

Hormone replacement therapy involves the oral intake of estrogen and sometimes progestin to maintain estrogen levels following menopause or a hysterectomy. There are currently 40 million post-menopausal American women, and population trends suggest that another 20 million women will experience menopause in the next decade (Andrews 1995). It has been estimated that of the 40 million post-menopausal women, 5 to 13 million are prescribed hormone replacement drugs (Andrews 1995). Premarin, a hormone replacement drug manufactured by Wyeth-Ayerst was first on the list of top selling retail drugs in 1995 (Glaser 1996). Approximately 80 to 90% of the prescriptions filled for Premarin were prescribed to relieve post-menopausal symptoms such as hot flashes, while the other 10 to 20% were prescribed for prevention of osteoporosis (Glaser 1996). The active estrogenic component of hormone replacement drugs are primarily the conjugated equine estrogens, as well as conjugated estrone and 17α- and 17β-estradiol (Bhavnani and Woolever 1991). The regimen varies depending on the individual circumstance, but typically involves daily intake of 0.625 mg of conjugated estrogens for 25 consecutive days, followed by five days with no drug treatment.

Growth Enhancement

Since the synthesis of DES in the 1940s, the use of inexpensive and potent synthetic steroids have been used in feed livestock to promote growth and improve feed efficiency. The use of growth enhancing products in the United States is regulated by the Food and Drug Administration (FDA). In 1995, there were 23.4 million feedlot cattle in the United States sold for slaughter (USDA 1996). It has been estimated that at least 90% (21.4 million) of feedlot cattle slaughtered in 1995 were administered growth enhancing hormones. Estradiol and estradiol benzoate are often used in conjunction with androgens (trenbolone acetate, testosterone propionate) and progestins (progesterone) in growth enhancement products. Cattle are administered growth enhancing hormones in a one time dose at time of first feed by means of a subcutaneous ear implant. The dose of estrogen administered ranges from 20-45 mg.

Estimated Quantities of Pharmaceutical Products Prescribed Annually

Given the number of women prescribed oral contraceptives and hormone replacement therapies in the United States, as well as the number of feedlot cattle administered growth enhancing hormones, the amount of estrogens used annually were estimated (Table 2). The estimated kilograms per year of ethinyl estradiol, conjugated estrogens and estradiol used in oral contraceptives, hormone replacement therapy, and growth enhancement products, respectively, were calculated by multiplying the number of users by the mean daily dose and the days per year used. A sample calculation to estimate the amount of ethinyl estradiol used annually in the United States is provided in Figure 2.

Table 2 includes 1995 retail sales (kg/year) of pharmaceutical estrogens (IMS America 1995). The kg/year of pharmaceuticals estrogens sold by retail outlets provided a comparative value for the estimated annual quantity of estrogens used. Estimates of hormone replacement therapies were very similar to the retail sales data provided by IMS America. However, retail sales data for ethinyl estradiol were much lower than the estimated used quantity. This discrepancy may be explained by the fact that retail sales data do not include oral contraceptives distributed by nation-wide health clinics such as Planned Parenthood or other non-retail sources. Although the number of women receiving oral contraceptives from family planning services is not known, over 6.5 million American women in 1994 used subsidized clinics for contraceptive purposes (Frost 1996). Data were not available on retail sales volume of livestock growth enhancement products.

Expected Introduction Concentration

As part of the environmental assessment for all human drugs, pharmaceutical companies are required by the FDA to calculate an expected introduction concentration (EIC) of products to the aquatic environment as a result of patient use (CDER 1995). The EIC is an initial calculation used to determine if further environmental assessment is warranted. As such, the EIC does not typically consider metabolic processes.

The EIC for ethinyl estradiol can be calculated by dividing the amount of product produced each year by the liters per day entering sewage treatment facilities. Since production numbers were not available, the estimated kilograms of estrogens sold and used each year were utilized. A sample calculation of the EIC for ethinyl estradiol is presented in Figure 2. The EIC was calculated using 1992 Environmental Protection Agency (EPA) data on the amount of waste water entering publicly owned treatment works in the United States (4.07×10^{13} liters per year) and the estimated kilograms per year of ethinyl estradiol used in oral contraceptive formulations (88 kg/year). Therefore, from the present calculations, 2.16 ng/L of ethinyl estradiol has the potential to reach sewage treatment facilities. Table 3 presents the EIC of ethinyl estradiol, conjugated estrogens, and estradiol from growth enhancement hormones to the aquatic environment based on the estimated annual use, as well as available 1995 kg/year retail sales data. Again, the lower EIC derived sales volume for ethinyl estradiol can be attributed to the exclusion of sales by other non-retail distributers of oral contraceptives. The EIC of hormone replacement drugs calculated from prescribed volumes was reasonably consistent with the EIC calculated from retail sales data. The EIC of conjugated estrogens was greater than the EIC for ethinyl estradiol. This was expected as not only are more women prescribed hormone replacement drugs, but the dosage is much greater.

Significance of EIC

Investigations conducted in the United Kingdom quantified ethinyl estradiol in sewage effluent at 0.2 to 7.0 ng/L (U.K. Environment Agency 1996). As little as 2 ng/L of ethinyl estradiol has been shown to induce vitellogenin and inhibit testicular growth in male rainbow trout (Jobling et al. 1996). Therefore, it is quite possible that pharmaceutical products enter the aquatic environment in concentrations sufficient to elicit estrogenic responses. In addition, hormone replacement therapies consisting of conjugated equine estrogens, estradiol, and estrone may represent significant loadings to the environment. The U.K. Environment Agency has reported concentrations of estradiol and estrone as high as 40 ng/L in final sewage effluent (U.K. Environment Agency 1996). In the present discussion, the EIC calculated for estradiol (14.2 ng/L) was based solely upon the use of growth enhancing hormones used in feed livestock. This figure (14.2 ng/L) does not include the endogenous estrogens present in hormone replacement therapy and those that are naturally excreted from reproductive age women. Although these endogenous compounds are less potent than the synthetic steroids, they are likely to be present in larger amounts. The calculation of the EIC assumes that all drug taken is excreted from the body unchanged. It also assumes that no further degradation of estrogen occurs. However, both metabolism and biodegradation can decrease the EIC estimates.

METABOLISM AND EXCRETION OF NATURAL AND SYNTHETIC ESTROGENS

Metabolism of Natural Estrogens

17β-estradiol. Estradiol is released from the ovaries and oxidized to estrone in a reversible reaction catalyzed by 17-hydroxysteroid hydrogenase to estrone (Figure 3). Estrone is subject to metabolism by 16α-hydroxylase to produce 16α-hydroxy-estrone and subsequently estriol. Estrone can also undergo 2-hydroxylation to yield 2-hydroxy-estrone followed by 2-methoxy-estrone.

Bioavailability is determined, in part, by the amount of compound bound to plasma proteins and rate of metabolism. Estradiol and other endogenous estrogens exist in the blood bound to sex hormone binding globulin (SHBG), although a small percentage may bind with plasma albumin. Only the unbound estrogen is biologically active. Hepatic metabolism occurs very rapidly and plasma half life is measured in minutes (Williams and Stancel 1996).

The major metabolites of 17β-estradiol are excreted in urine, with estriol being the major metabolite; sulfate and glucuronide metabolites are also present in the urine (Williams and Stancel 1995). Radiolabelled studies

demonstrate that 80% of a dose is recovered in urine. Within the first few hours of administration, 50% of the compound is present in bile. However, only 7% is ultimately excreted in feces, demonstrating a large degree of enterohepatic circulation (Orme et al.1983).

Endogenous estradiol is excreted daily by women, and the amount depends on age, reproductive status, and day of the menstrual cycle. For example, a normal female typically excretes 25 to 100 µg/ day of estradiol at ovulation and 10-80 µg/day during the luteal phase. After menopause, the amount of estradiol excreted drops to 5 to 10 µg, whereas a pregnant women may release as much as 30 mg. Men also excrete approximately 2 to 25 µg of estradiol daily (Williams and Stancel 1996).

Metabolism of the Conjugated Estrogens

Equine estrogens and estradiol. Conjugated estrogens (mostly sulfate esters) undergo hydrolysis in the gut to remove the sulfate group and allow absorption by the gastrointestinal tract. The first pass metabolic effect is complete and similar to that of estradiol metabolism. However, the B ring of the equine estrogens is unsaturated and has a lower tendency to bind to plasma proteins, thereby enhancing bioavailability and action at the target cell (Bhavnani and Woolever 1991).

Metabolism and Excretion of Synthetic Estrogen

Ethinylestradiol. The ethinyl substitution at carbon 17 of ethinyl estradiol inhibits complete first pass metabolism. Bioavailability after the first pass effect is approximately 40% (Bolt 1977). Initial metabolism occurs in the gut where ethinyl estradiol is rapidly converted to ethinyl estradiol-sulfate. While ethinyl estradiol does not bind to SHBG, 97 to 98 % of ethinyl estradiol is bound to plasma albumin (Orme et al. 1983). Peak plasma concentration of ethinyl estradiol occurs within 2 hours of estrogen administration and the plasma half life varies between 6 and 20 hours (Orme et al. 1983). The relatively slow elimination from circulation enhances the effectiveness of the ethinyl estradiol.

Unlike estradiol, hydroxylation of ethinyl estradiol is most common at the 2 position yielding 2-hydroxy-ethinyl estradiol and 2-methoxy-ethinyl estradiol (Figure 4). Bolt (1977) reported that 29% of hydroxylation occurred at the 2 position. In some individuals, this number reached 64%. Hydroxylation can also take place at the 4, 6, and 16 position, although hydroxylation at these points are less common (Orme et al. 1983).

Both 2-hydroxy-ethinyl estradiol and 2-methoxy-ethinyl estradiol can be excreted as sulfate and glucuronide conjugates (Orme et al. 1983). In contrast to estradiol where the majority of metabolites are excreted in the urine, the ethinyl estradiol urinary and fecal metabolite ratio is approximately 4:6, with a 91% recovery rate (Speck et al. 1976). The majority of radioactive material recovered in the urine is glucuronidated (Maggs and Park 1985), with about 10% attributed to sulfate conjugation. Conjugation takes place at the carbon 3 position creating susceptibility to enterohepatic circulation. Earlier investigations by Kulkarni and Goldzieher (1970) and Kamyab et al. (1969), cited in Orme et al. (1983), reported that 1 to 16 % of the ethinyl estradiol was found in unchanged form in the feces. Orme et al. (1983) suggested that the presence of unchanged compound may reflect deconjugation in the colon. Mestranol, a 3-methyl ester of ethinyl estradiol, is inactive until rapidly demethylated to ethinyl estradiol in the body. Its metabolic derivatives are similar to that of ethinyl estradiol.

Metabolism and Excretion of Estrogens in Livestock

As previously mentioned, a number of feed livestock receive growth enhancing hormones as estrogen, progesterone, and androgens. For example, estradiol benzoate in conjunction with trenbolone acetate (testosterone derivative) is used for increased weight gain and feed efficiency. Estradiol benzoate is metabolized *in vivo* to 17-β estradiol, estrone and related conjugates (Syntex Animal Health 1995). It has been estimated that 28 mg of estradiol benzoate has equal potency to approximately 20 mg of estradiol (Syntex Animal Health 1995). Ivie et al. (1986) examined the fate and tissue residues of radiolabelled 17β-estradiol in Holstein steer calves. Radiolabelled compound was detected in urine and feces 24 hours after an intramuscular injection; greater amounts were found in the feces than in urine. Approximately 57% of the total dose was recovered in feces and 42 % in urine (Ivie et al. 1986).

ENVIRONMENTAL FATE

Biodegradation in the Environment

Radiolabelled studies in humans and animals indicate that much of the ingested dose of a synthetic estrogen is excreted in urine and/or feces as a conjugate while little of the product is excreted as parent compound. However, it is apparent that deconjugation occurs in the human colon suggesting that similar metabolic events, involving micro flora, may occur in sewage treatment facilities. For example, synthetic as well as natural estrogens found in sewage effluent in the United Kingdom were detected as "free" biologically active compounds (U.K. Environment Agency 1996).

Activated sewage sludge contains microbial species capable of degrading both natural and synthetic steroid compounds.

Several studies have examined the biodegradation and concentration of natural and synthetic steroids in activated sewage and waste water effluent. Culture enrichment studies have been conducted in which natural and synthetic estrogens were incubated with micro flora from activated sludge and the percent loss of steroids was measured over 28 days (Tabak and Bunch 1969). Steroids were introduced into the culture enrichment system using two methods, and in either case synthetic estrogens (ethinyl estradiol, mestranol) were more resistant to microbial degradation than natural steroids (estrone, estradiol, estriol). Higher biodegradation was found in enrichment studies when steroid was suspended in the media rather than coated on the bottom of the flask. Similar findings were reported by Tabak et al. (1981) where concentrations of natural and synthetic steroids were measured in raw and treated sewage from 14 sewage treatment plants in Cincinnati, Ohio (US). Higher concentrations of synthetic steroids were measured in both the raw and treated sewage when compared to concentrations of the endogenous estrogens. For example, the mean concentration of ethinyl estradiol detected in raw and treated sewage was 1.21 ng/L and 0.81 ng/L, respectively. In comparison, estradiol, estrone, and estriol were detected in raw and treated sewage in concentrations ranging from 0.01 to 0.08 ng/L. Figure 5 illustrates the greater resistence to degradation of synthetic hormones in comparison to the natural hormones analyzed by Tabak et al. (1981) following primary and secondary treatment. These data suggest that estrogenic compounds have the potential to enter receiving waters.

SUMMARY AND CONCLUSION

The available evidence suggest that potent estrogens have the potential to reach the aquatic environment in concentrations sufficient to elicit estrogenic responses by means of point and non-point sources such as municipal and industrial sewage outfalls. While the majority of the compounds prescribed are excreted in conjugated form, studies have shown that deconjugation can occur in the human colon. Thus, it is plausible that a similar mechanism to convert compounds back to their active form could exist in the micro flora of sewage treatment facilities. This is supported by reports of unbound ethinyl estradiol, estradiol, and estrone quantified in the sewage effluent studies in the United Kingdom (U.K. Environment Agency 1996).

Estradiol and its metabolic products, although apparently more readily biodegradable, may also pose concern, especially in relation to immediate discharge to rivers and streams near agricultural areas. Concern is also warranted with regards to the potential discharge of conjugated estrogens to the aquatic environment. The equine estrogens, used in hormone replacement therapy, have similar potencies to that of the natural estrogens estradiol, estrone, and estriol. In light of the fact that estradiol, estrone, and estriol have been identified as estrogenic components of sewage effluent (U.K. Environment Agency 1996) further investigations should be aimed at determining the presence of the equine-derived estrogens in the environment.

To address the research issues presented above in relation to the Southeastern United States, we are currently conducting an investigation, supported by the Mississippi Water Resources Research Instituute, with the overall goal of assessing environmental estrogens in wastewater. Specifically, during the first year of study biological screening of wastewater for estrogenic activity is being conducted at several locations using a caged channel catfish model. In a concurrent collaborative effort, investigators at the University of Tennessee are investigating the microbial transformation of steroid hormones modulating the fate of these compounds and their activity as environmental estrogens.

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Table 1. Human use of estrogenic pharmaceutical products.

Human Use	Treatment	Active Estrogen
Oral Contraceptives	ovulation inhibition	ethinyl estradiol and mestranol
Hormone Replacement Therapy	menopause osteoporosis hypogonadism	conjugated estrogens
Fertility	ovulation induction	clomiphene (anti-estrogen)
Cancer Chemotherapy	breast and prostate cancer	tamoxifen (breast cancer) diethylstilbestrol (prostate cancer)

Table 2. Kilograms of estrogenic pharmaceuticals prescribed and sold to retail pharmacies.

Estrogenic Pharmaceutical Products	Estrogens used (kg/year)[a]	Retail sales (kg/year)[b]
Oral Contraceptives -ethinyl estradiol, mestranol	88	0.4887
Hormone Replacement Therapy -conjugated estrogens	1687.5	1500
Growth Enhancing Hormones -estradiol	579.2	NA

NA, not available
[a] calculated from the number of users and average doses.
[b] from IMS America.

Table 3. Expected Introduction Concentrations (EIC) of estrogenic pharmaceuticals to the aquatic environment.

Pharmaceutical Product	EIC from estimated use (ng/L)	EIC from retail sales (ng/L)
Oral Contraceptives - ethinyl estradiol	2.16	0.012
Hormone Replacement Therapy -conjugated estrogens	41.5	36.9
Growth Enhancing Hormones -estradiol	14.2	NA

NA, not available

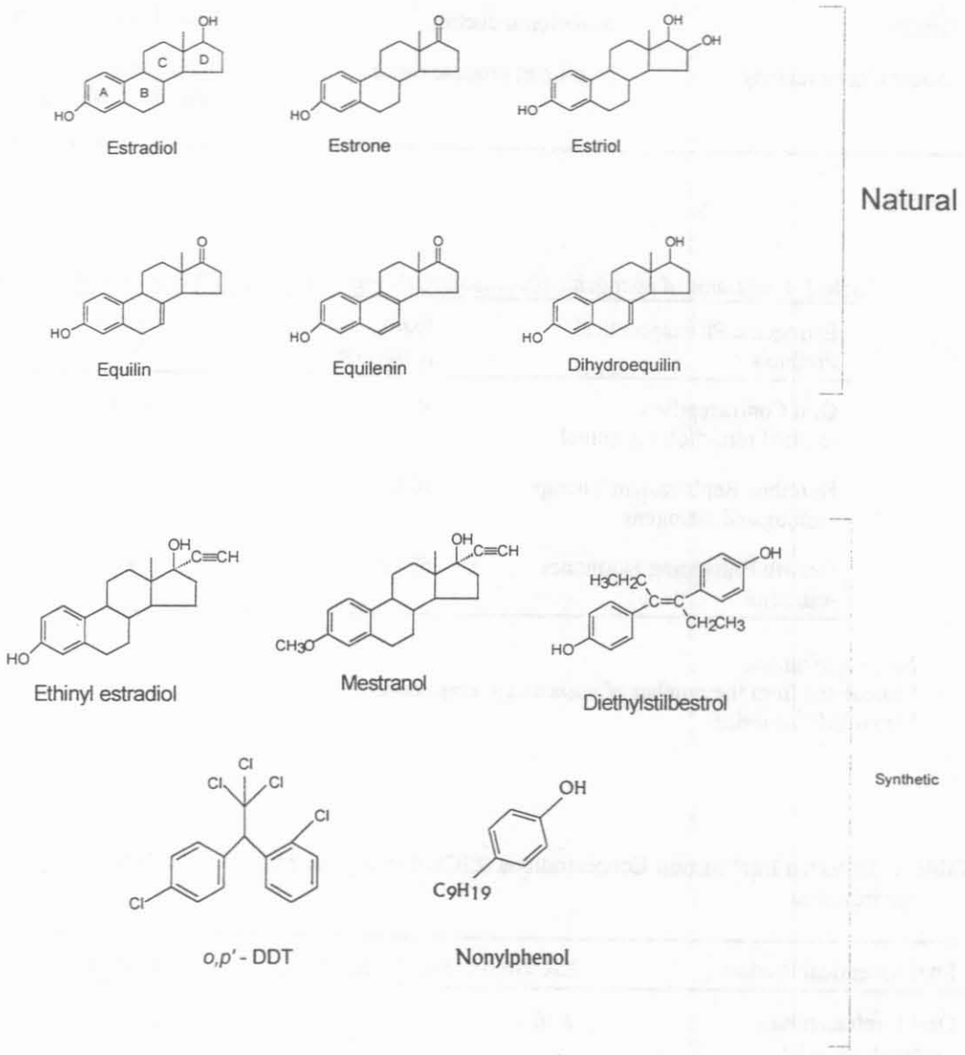

Figure 1. Chemical Structures of Natural and Synthetic and Environmental Estrogens.

EIC of Ethinyl Estradiol to the Aquatic Environment

$$\frac{\text{kg / year prescribed}}{\text{liters / year entering POTW}} = \frac{\text{88 kg / year}}{4.07 \times 10^{13} \text{ liters/year}} = \mathbf{2.16\ ng / L}$$

where,

kg/year prescribed = 35 ug ethinyl estradiol x 10 million users x 252 days

liters per year entering POTW = 1.115×10^{11} liters/day x 365 days

Figure 2. Calculation of the Expected Introduction Concentration of Ethinyl Estradiol to the Aquatic Environment.

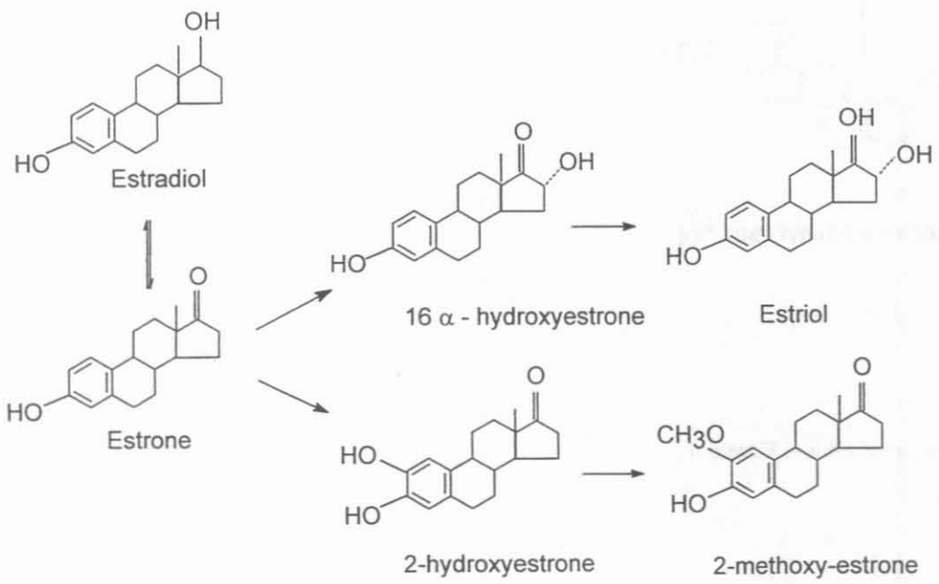

Figure 3. Metabolism of Estradiol

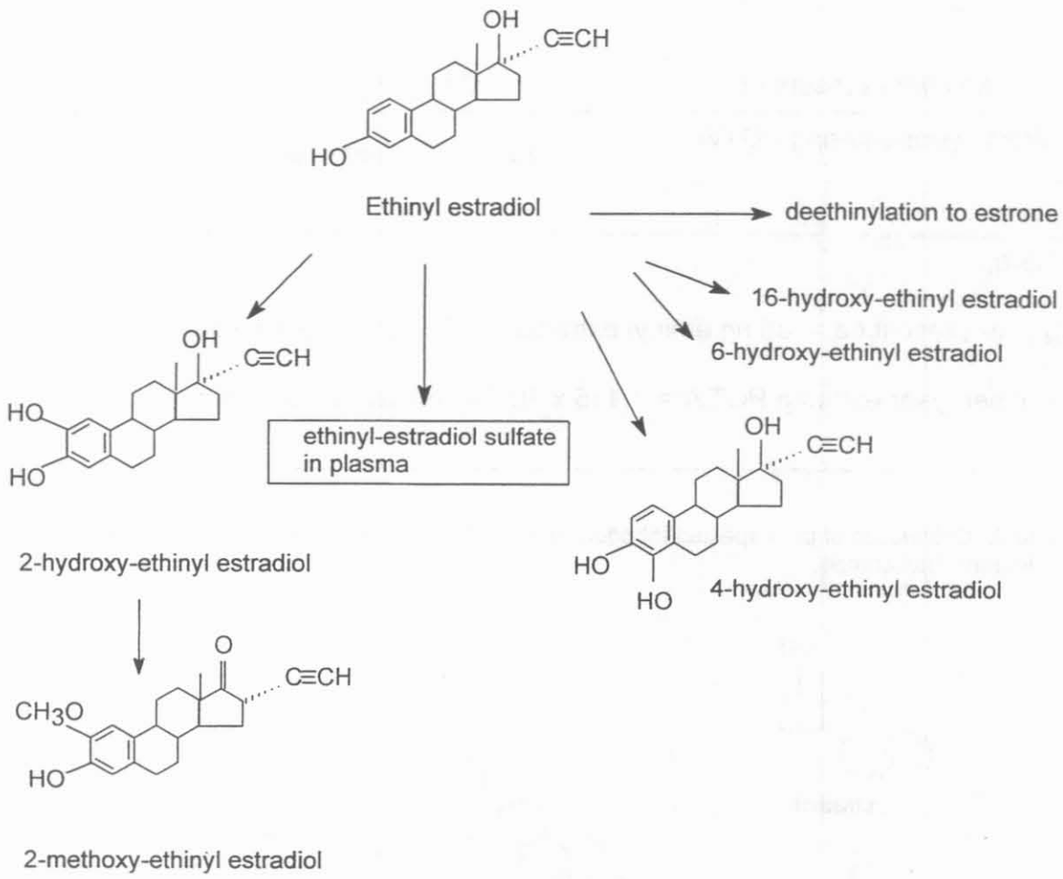

Fig 4. Metabolism of Ethinyl Estradiol

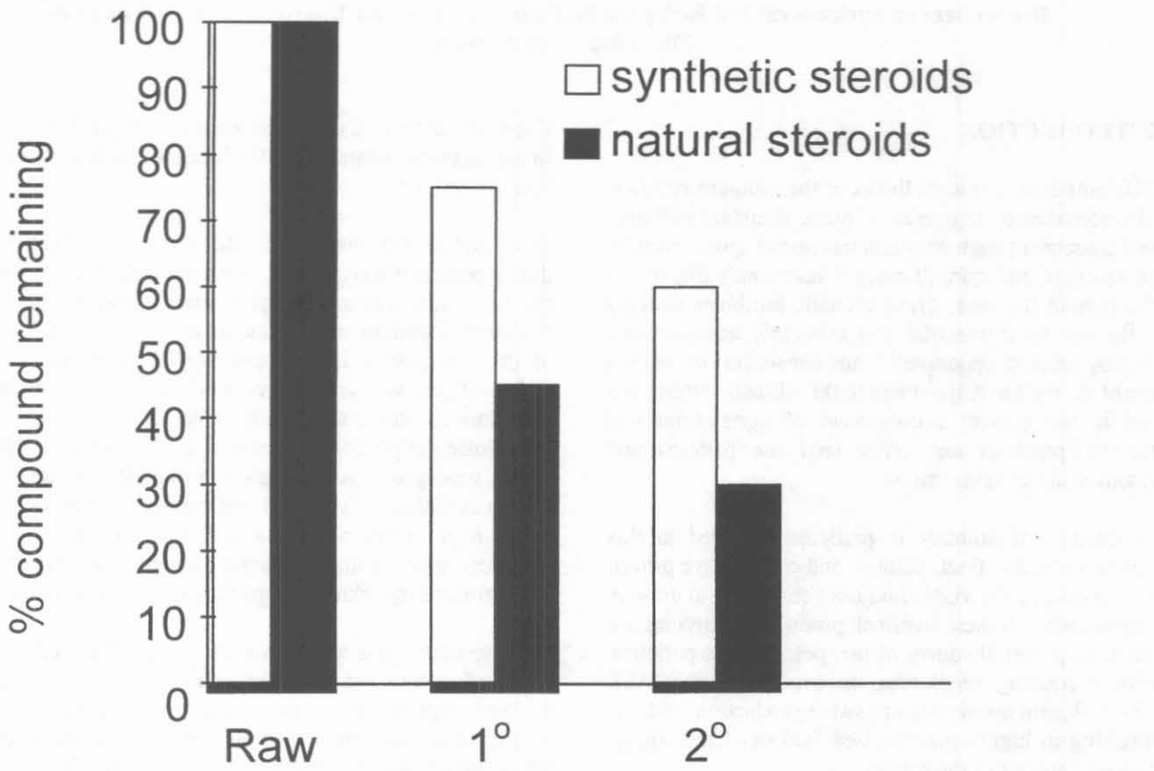

Figure 5. Biodegradation of natural and synthetic estrogens in raw and treated sewage.
Data adapted from Tabak et al. (1981) demonstrating the mean percent of natural and synthetic steroids remaining after primary and secondary treatment.