CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Nile tilapia

Nile tilapia is a freshwater fish of the *Cichlidae* order, *Oreochromis* genus and *Oreochromis niloticus* species (see Figure 2.1). Nile tilapia has become increasingly popular for fish farmers in Thailand because of its high market value. Moreover, Nile tilapia are normally more tolerant in a wide range of environmental conditions such as high salinity, high water temperature, low dissolved oxygen, and high ammonia concentrations than other farmed freshwater fish. They are easily spawned, grow rapidly and can be fed with a wide range of fish feed including natural and artificial foods (Popma and Masser, 1999). In Thailand, three types of ponds are used for farming Nile tilapia: concrete ponds, earthen ponds and floating baskets (nylon pot) in river. Some of the issues faced by fish farmers in a mixed-sex fish farm are the difference in weights and sizes of the fish produced. This is generally attributed to overpopulation in the ponds and loss of energy from reproduction. Therefore, fish farmers favor culturing monosex (all male) population.

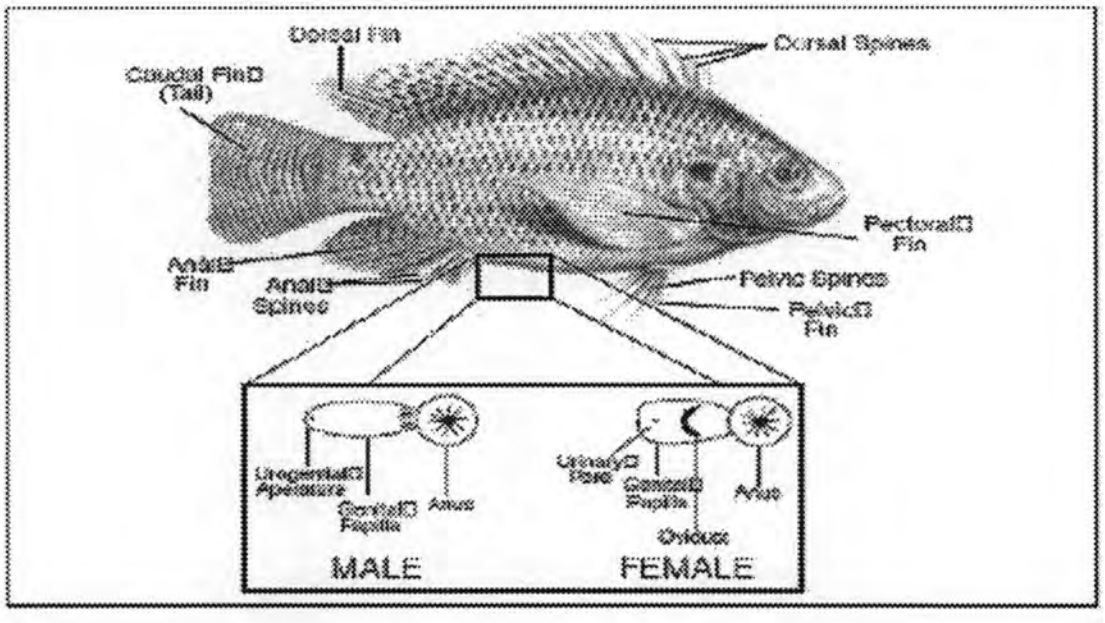


Figure 2.1 Typical Nile tilapia and various body features

2.2 Masculinization of Nile tilapia using 17alpha-methyltestosterone

There are four masculinizing techniques: manual sexing, interspecies hybridization, genetically altered male tilapia (supermale tilapia) and hormone-induced sex reversal (วิเชียร, 2542). Among these techniques, hormone-induced sex reversal is the most efficient, easy to implement and less expensive than other techniques. 17alpha-methyltestosterone (MT), an anabolic androgenic male steroid hormone, is normally used to induce sex reversal and produce an all male population. Two methods are used in the masculinization of Nile tilapia using MT. The first method is oral administration by feeding tilapia fry with MT-impregnated food. MT is mixed with fish feed at 60 µg of MT per kg of fish feed and fed to hatched fry after yolk sac absorption. The amount of food used for feeding the fry is dependent on the age of the fry. In the first week, the amount of food fed is about 30 % of their body weight. It is reduced to about 20 % and 15 % during the second and third week, respectively. By using this protocol, the percent of male tilapia in a musculinization tank is about 86-100 % (คีรี, 2542; เพ็ญพรรณ, 2547). The second method is a single immersion treatment of fry, 14 days after hatching, with MT at a concentration of 1800 µg/L for 48 hr. This method gives a male tilapia population of 90 % (Gustavo and Luis, 2003). Although masculinization of tilapia may increase the profits of commercial aquaculture farmers, release of MT from aquaculture farms can impact human and animal health. Because MT is classified as an endocrine disrupting compound (EDC) (Andersen *et al.*, 2006), MT can interfere with normal endocrine system and reproductive system in humans and animals (Bhandari *et al.*, 2006; Korsgaard, 2006; Schulte-Oehlmann *et al.*, 2004; Selzsam *et al.*, 2005). Many researchers have shown the effect of MT on aquatic wildlife (Korsgaard 2006). Furthermore, MT has been identified as a probable carcinogenic substance (Roberts and Essenhigh, 1986; Overly *et al.*, 1984).

2.3 Masculinization ponds of Nile Tilapia fry

There are 3 types of ponds for masculinizing Nile tilapia fry. The first is a rectangular-shaped earthen pond with an area of about 50-1,600 m^2. The depth of the water is about 1 m. This type of pond is the most effective in terms of productivity because the conditions in this pond are similar to that of a natural pond. The second is

a rectangular or round cement pond, with an area of more than 10 m^2. The depth of the water is approximately 80 cm. For this type of pond, an aerator is needed to provide oxygen and to increase the production of tilapia. However, the cost of this type of pond may be more expensive than the earthen ponds because of the materials of construction and size which would require several ponds. The third type is to grow the fry within an enclosure or cage made of nylon net placed inside a earthen pond, natural pond, or natural receiving water. The size of the nylon cage is approximately 5 m x 8 m x 2 m, with the depth of the water inside the net at about 1 m. Pillars are typically used to support the net at 4 corners.

2.4 Anabolic androgenic steroids

Anabolic androgenic steroids are a class of steroids that interact with androgen receptors to increase muscle and bone synthesis. A steroid is a lipid characterized by a carbon skeleton with four combined aromatic rings. All steroids are derived from the cholesterol or the acetyl CoA biosynthetic pathway (Stryer, 1996) and different steroids vary in the functional groups attached to these rings.

There are natural and synthetic anabolic steroids. Examples of natural steroids are testosterone and androstenedione and synthetic steroids are methyltestosterone and 17β-trenbolone (Bauer, 2002). Testosterone and methyltestosterone are two common anabolic steroids used to enhance performance. The focus of this study is on methyltestosterone.

2.4.1 17alpha-methyltestosterone

MT is an oral form of testosterone which is a naturally occurring androgen ("male" sex hormone) that is produced in the testes of men and, in small amounts, by the ovaries and brains in women. MT is a synthetic derivative of testosterone with a methyl group added to the C-17-alpha position of the molecule. MT is a hormone used to treat men with testosterone deficiency and also used in women to treat breast cancer, breast pain, swelling due to pregnancy, and with estrogen to treat symptoms of menopause. MT is associated with less hepatic metabolism following oral administration when compared to testosterone (Murad and Haynes, 1985).

2.4.2 Structure of 17alpha-methyltestosterone

The chemical name of MT is 17beta-hydroxy-17alpha-methylandrost-4-en-3-one. The synonyms of MT are methyltestosteronum and methyltestosteronum. In addition MT is sold under commercial names such as Android® and Virilon®. The main structure of MT consists of three aromatic rings and one penta-cyclic ring as shown in Figure 2.2. 17alpha-methyltestosterone is structurally similar to testosterone with a methyl group bonded to the main structure at C17 position.

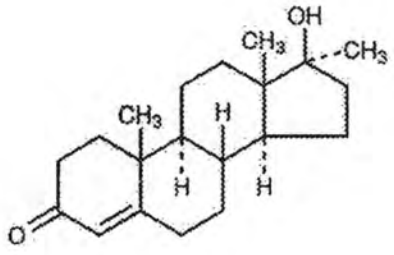


Figure 2.2 Chemical structure of MT

2.4.3 Physicochemical properties of 17alpha-methyltestosterone

The physicochemical properties of MT are shown in Table 2.1. The water solubility of MT is about 3.39 mg/L at 25 °C and the log K_{ow} is approximately 3.36 indicating that MT has a tendency to be sorbed onto soils or sediments rather than be dissolved in water.

2.5 Adverse effect of 17alpha-methyltestosterone

2.5.1 Definition of endocrine disrupters

The endocrine system consists of several glands which are ductless and produce hormones for different functions. The hormones are transferred through the bloodstream and are responsible for stimulating the natural response of the target organ. When a hormone reaches the target cell consisting of a binding site (receptor) and an effecter site, the hormone binds with the receptor site and then the effecter site is altered which results in the response.

There are many definitions for endocrine disrupters. The "Weybridge" definition of endocrine disrupters from the European Workshop on "The impact of

endocrine disrupters on human health and wildlife" (European Commission, 1996) is stated as follows.

"An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, subsequent to changes in endocrine function."

The U.S. Environmental Protection Agency (USEPA) has proposed a more detailed definition of endocrine disrupters (USEPA, 1997):

"An endocrine disrupter is an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior."

The endocrine disruptor compounds can be classified into 5 types: steroid compounds (e.g., estrogens and testosterones); surfactants (e.g., nonylphenol and its ethoxylates); pesticides, herbicides and fungicides (e.g., DDT, dieldrin, 2,4-D, tributyltin); polycyclic aromatic compounds (e.g., PAHs, PCBs, brominated flame retardants), and organic oxygen compounds (e.g., phtalates, bisphenol A).

Table 2.1: Physiochemical properties of MT

Properties	Value
Name	17α-methyltestosterone
Formula [a]	$C_{20}H_{30}O_2$
CAS number	58-18-4
Molecular weight	302.46
Color/Form [b]	White or creamy white crystals or crystalline powder
Odor [b]	Odorless
Melting point [a]	166-166 °C
water solubility	3.39 mg/L at 25 °C
log K_{ow} [c]	3.36
vapour pressure	1.85×10^{-8} mmHg at 25 °C
Henry's law constant	4.68×10^{-9} atm.m^3/mol at 25 °C
Hydroxyl radical reaction rate constant	1.0×10^{-10} cm^3/mole-sec at 25 °C
Ozone reaction rate constant	1.1 x 10 cm /mole-sec at 25 °C

[a] O'Neil, 2001; [b] Lewis, 1997; [c] Yalkowsky and He, 2003

2.5.2 Mechanistic activity of endocrine disrupters

Hormones have a specific and high affinity for the specific receptor site, and a small amount of hormone is sufficient to induce the response (Figure 2.3 (a)). However, low concentrations of an endocrine disruptor compound can bind to the receptor sites causing an effect and eliciting a response. The response can be either an agonistic effect or an antagonistic effect. For an agonistic effect (Figure2.3 (b)), the endocrine disruptor compound mimics the hormone and binds to the hormone receptor site and induces a response. For an antagonistic effect (Figure 2.3 (c)), the endocrine disruptor compound acts as the hormone blocker binding to the hormone receptor site and inhibiting the response or preventing the work of the natural hormone (Birkett, 2003).

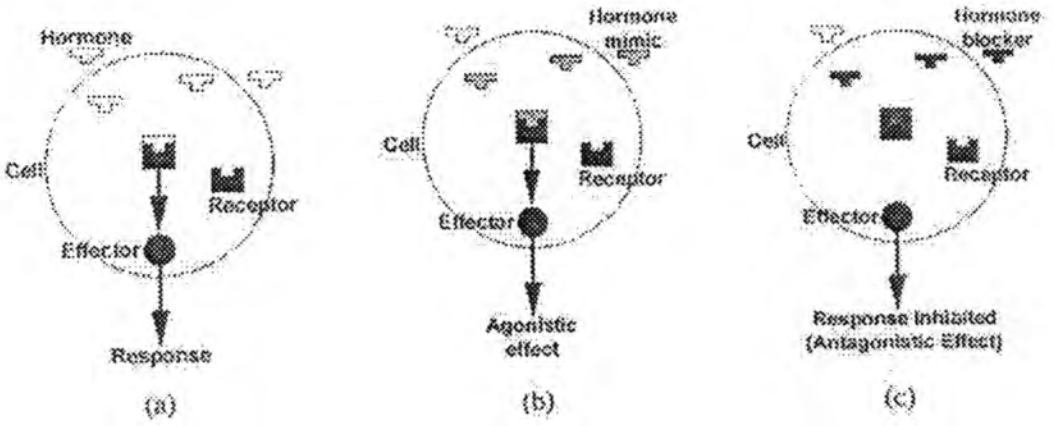


Figure 2.3: Endocrine disruption processes (a) natural response; (b) agonistic effect; (c) antagonistic effect (Birkett, 2003)

2.5.3 Effect of 17alpha-methyltestosterone on living organisms

MT can act as an endocrine disruptor compound by interfering with the normal function of the endocrine and reproductive systems (Andersen *et al.*, 2006). In the ovary, steroid enzymes such as cytochrome P450 cholesteral-side-chain-cleavage (P450scc), 3β-hydroxy-steriod (3β-HSD) and P450 aromatase (P450arom) are involved in the ovarian process in females and the lack of these steroid enzymes will result in a masculinizing effect (Bhandari *et al.*, 2006). Protein vitellogenin (vtg) is another compound or indicator that expresses the female characteristic. When exposed to 10-500 ng/L of MT for ten days, the circulation yolk-precusor protein

vitellogenin was found to decrease in female eelpout (*Zoarces viviparous*) (Korsgaard, 2006). In a study by Schulte-Oehlmann *et al.* (2004), imposex which is the development of male sex organ such as penis and vas deferens was stimulated in female freshwater ramshorn snail (*Marisa cornuarietis*) when exposed to 0.1-1.0 ug/L of MT for six months. MT not only affects the aquatic life and invertebrates near the masculinzing pond but it can also impact birds. The egg-laying rate of female Japanese quails (*Coturnix cotumix japonica*) and the fertility rate in male Japanese quails were found to decrease when exposed to 50-110 mg/L of MT for 3 weeks (Selzsam *et al.*, 2005). Additionally, Hulak *et al.* (2008) studied the effect of residual MT in the water of recirculation system from the masculinization process. They found that the residual MT in the recirculated water can induce sex inversion of common crap progeny by as much as 81-100%, even though the recirculated water has been treated by a biological filter. Moreover, they found that MT can inhibit the production of testosterone hormone in common crap as in rainbow trout (Fitzpatrick *et al.*, 1993). Kang *et al.* (2008) studied the effect of MT on adult medaka (both male and female). They found that the fecundity and fertility of paired medaka were significantly decreased when exposed to MT concentration of more than 46.8 ng/L. This concentration can also inhibit gonadal development and adversely affect the reproduction of medaka. When exposed to an overdose of MT at about 20,200 µg/L, aromatase enzyme in animals can convert MT to estrogen which in turn can impact the male wildlife (Fitzpatrick, *et al.*, 1999).

2.5.4 Carcinogenicity

Anabolic steroids may be carcinogenic substances. They can stimulate growth of sex-hormone dependent tissue, primarily the prostate gland in men. Precocious prostatic cancer has been described after long-term anabolic steroid abuse (Roberts and Essenhigh, 1986). Cases where hepatic cancers have been associated with anabolic steroid abuse have been reported (Overly *et al.*, 1984). MT has been doubted as a carcinogenic compound (Soe *et al.*, 1992; Nakata *et al.*, 1997)

2.5.5 Non-carcinogenicity

The adverse effects of anabolic steroids include weight gain, fluid retention, and abnormal liver function as measured by biochemical tests. Administration to

children can cause premature closure of the epiphyses. Men can develop impotence and azoospermia. Women are at risk of virilization (Dewhurst and Gordon, 1984; Ferner and Rawlins, 1988; Kennedy, 1992; Ross and Deutch, 1990; Ryan, 1981; Wagner, 1989). MT is known as a synthetic compound that can induce adverse effect on human and animal's health.

2.6 Occurrence of MT in environment

During masculinization, fry are fed with fish feed containing 60 mg of MT per kilogram of fish feed. The dose is effective in converting the sex of Nile tilapia. MT in fish feed can leak into the environment as presented in Contreras-Sánchez *et al.* (2001) study. They found the concentration of MT in pond water increased to 100 ng/L one minute after the MT-impregnated food was added and up to 160 ng/L after 15 minutes. Recent researches have found residual MT accumulating in water and sediments in masculinization ponds. Contreras-Sánchez *et al.* (2002) found that during the masculinization process, MT was not detected in the water samples, but MT was found in the sediment with concentrations varying from the lower limit of detection to 368.9 pg/g. Fitzpatrick and Contreras-Sánchez, (2000) found that MT persisted in the sediment at a concentration of approximately 2.8-2.9 ng/g up to eight weeks after the end of treatment in a model masculinization pond. Similarly, MT concentrations from fish farms in Mexico were found to be about 4.6 ng/g in the sediment of the masculinizing ponds (Contreras-Sánchez *et al.*, 2001). These results indicate that MT is sorbed to the sediment with the sediment acting as a sink for MT.

2.7 Fate of MT in the environment

Although, MT is widely used for masculinization purposes in aquaculture technique throughout the world, literature on the fate of MT in the environment is limited. However, the structure and properties of MT are similar to that of other steroids such estrogens and testosterone. Therefore, this review will cover the fate of estrogens, both natural estrogens: estrone, estradiol and estriol and synthetic estrogens: ethynylestradiol and testosterone which may provide information on possible fate of MT in the environment.

2.7.1 Sorption

Sorption is an important process affecting the fate of MT in the environment. MT has high log K_{ow} indicating that MT tends to be sorbed onto solid phase rather than be present in the dissolved phase. A recent study by Chotsukarn *et al.* (2008) showed that sorption of MT was a function of organic carbon in the soil and sediment with log K_{ow} value of MT of about 3.36 which is similar to the log K_{ow} value of estrogens and testosterone in the range of 2-4 and 3.22, respectively (Table 2.2). Lee *et al.* (2003) conducted batch experiments to study the sorption of estradiol, ethylnylestradiol and testosterone in soils and sediments. They found that estradiol can be rapidly sorbed on soils and sediments within a few hours. In addition, Lai *et al.* (2000) found that estrogens can be rapidly sorbed onto soils and sediments within the first-half hour followed by slow sorption. However, many factors can affect sorption of hormone onto soil and sediment such as organic content, surface area, salinity and cation exchange capacity. Lai *et al.* (2000) found that sorption of estrogens increased for soils with higher organic content. Van Emmerik *et al.* (2003) compared the sorption of estradiol to goethite (iron oxide) and clay minerals and found that sorption of estradiol onto clay occurred is a 2-step process with rapid sorption on the superficial surface of clay followed by slow sorption by diffusion into intra-aggregated micropores. For goethite, estradiol was found to be sorbed only on the surface of goethite and was easily desorbed into aqueous phase. Lai *et al* (2000) studied the effect of salinity on sorption of estrogens and found that sorption of estrogens increased when the salinity was increased, suggesting that estrogens can be highly accumulated in sediments of estuarines and marine areas. Estradiol can be transformed into estrone whereas testosterone can be transformed into androstenedione and an unknown transformed product (Lee *et al.*, 2003).

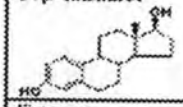
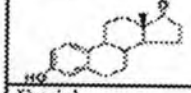
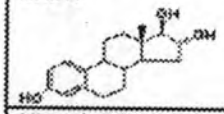
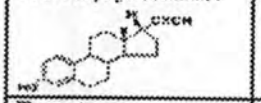
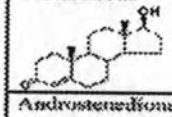
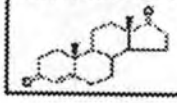
2.7.2 Biodegradation

2.7.2.1 Biodegradation by mixed culture

Not much is known about the biodegradation of MT. However, studies on the biodegradation of steroids such as estrogens may provide information on possible biodegradation of MT. Many studies are on the biodegradation of estrogens in activated sludge of wastewater treatment plants. In general, under aerobic conditions,

natural estrogens (estrone, estradiol and estriol) are degraded by microorganisms in activated sludge whereas ethynylestradiol, a synthetic estrogen, has been found to persist in contact with activated sludge (Ternes *et al.*, 1999). Estradiol was found to transform to estrone in activated sludge (Ternes *et al.*, 1999) and the by-products from the biodegradation of estrone were not observed (Ternes *et al.*, 1999). Vader *et al.* (2000) studied the biodegradation of ethynylestradiol by nitrifying activated sludge

Table 2.2: Property of estrogens and androgens

Hormone	Molecular weight (g mol^{-1})	Water solubility (mg L^{-1})	Log K_{ow}	MP °C
17β-estradiol 	272.4	13[a]	4.01[c] 3.10[a], 3.94[b]	173
Estrone 	270.4	13[a]	3.43[a], 3.13[a,b] 3.38[c], 2.45[f]	259
Estriol 	288.4	32[d]	2.81[a], 2.6[a] 2.55[c]	285
17α-ethynyl estradiol 	296.4	4.8	3.67[a], 4.15[b]	183
Testosterone 	288.4	18 - 25[b]	3.22[a]	155
Androstenedione 	286.4	37-41[e]	NA[g]	173-174

[a] Lai *et al.*, 2002; [b] Sagaya *et al.*, 2002; [c] Nuez and Yalkowsky, 1997; [d] Solubility from a control tablet comprising of estriol and alpha-cyclodextrin; [e] measured at 37 °C; [f] Suzuki *et al.*, 2001; [g] not available

(NAS) in batch experiments with different ammonia degradation rates and found that at high ammonia degradation rate (50 mg $NH_4^+g^{-1}$.DW.h^{-1}), ethynylestradiol was degraded but no degradation of ethynylestradiol was observed at low ammonia

degradation rate (1 mg $NH_4^+g^{-1}$. $DW.h^{-1}$). They suggested that ethynylestradiol was biodegraded by ammonia monooxygenase via cometabolism process. The degradation rate of estrone, estradiol, estriol and ethynylestradiol by NAS were found to be 0.056, 1.3, 0.03 and 0.035 hr^{-1}, respectively (Vader *et al.*, 2000). Shi *et al.* (2004) added ammonia oxidizing inhibitor to NAS and found that the degradation rate constant of ethynylestradiol reduced from 0.059 hr^{-1} (without inhibitor) to 0.0085 hr^{-1}. This suggested that ammonia oxidizing bacteria in NAS may be responsible for the degradation of ethynylestradiol.

Biodegradation of estrogens under anaerobic conditions have been found to be slower than under aerobic conditions. A study by Lee and Lui (2000) showed that about 80% of estradiol were degraded within 22 hours under aerobic conditions but only 50 % were degraded within 7 days under anaerobic conditions. There is only one study on the biodegradation of estradiol and ethynylestradiol under four different electron acceptors NO_3^-, Fe^{3+}, SO_4^{2-} and CO_2 (Czajka and Londry, 2006). In this study, $NaNO_3$, Fe(III)NTA, Na_2SO_4 and CO_2 were used for nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions. They found that ethynylestradiol persisted for every electron acceptor. Meanwhile, estradiol was found to degrade faster in iron(III)-reducing condition with a rate of about 325 ± 78 µg/L/d and followed by sulfate-reducing, methanogenic and nitrate-reducing conditions with rates of about 284 ± 31, 262 ± 40 and 180 ± 135 µg/L/d, respectively. Wattanodorn (2007) studied the degradation of MT by microorganisms in sediment under aerobic and anaerobic conditions and found that the degradation of MT under both conditions were faster under aerobic conditions than anaerobic conditions.

2.7.2.2 Biodegradation by pure culture

Ogura *et al.* (2004) isolated 7 strains of estradiol-degrading bacteria from soil with the strains divided into three species: *Rhodococcus rhodochrous*, *Sphingomonas koreensis* and *Sphingobium herbicidovorans*. In Ogura *et al.* (2004) study, estrone was observed to be formed with further degradation by these strains. Chao *et al.* (2004) isolated estradiol-degrading bacteria strain D12 from soil. Using 16s rDNA sequencing and phylogenetic analysis, the strain D12 was probably a new species in the genus of *Sphingomonas*. Strain D12 can degrade estradiol with a degradation rate of 0.12 hr^{-1}. As in Ogura *et al.* (2004) study, estrone was observed during

biodegradation and was further degraded by this strain. Yu *et al.* (2007) isolated 14 strains (KC1-14) of estradiol-degrading bacteria from activated sludge. The 14 strains, 8 genuses were identified: *Aminobacter* (strains KC6 and KC7), *Brevundimonas* (strain KC12), *Escherichia* (strain KC13), *Flavobacterium* (strain KC1), *Microbacterium* (strain KC5), *Nocardioides* (strain KC3), *Rhodococcus* (strain KC4) and *Sphingomonas* (strain KC8, KC11 and KC14). All strains were found to degrade estradiol to estrone through three different biodegradation patterns (A, B, and C). For pattern A, all strains except for strains KC6-8 can degrade estradiol to estrone but no biodegradation of estrone was observed. For patterns B and C, both estradiol and estrone were found to degrade. Biodegradation of both estradiol and estrone in pattern B by strains KC6 and KC7 was found to be more rapid than that of estradiol and estrone by strain KC8 for pattern C. Two other ethynylestradiol-degrading bacteria, *Sphingobacterium sp JCR5* (Haiyan *et al.*, 2007) and *Fusarium poliferatum strain HNS-1* (Shi *et al.*, 2002) were isolated from activated sludge and cowshed manure sample, respectively. Both species were found to use ethynylestradiol as a sole carbon source. In addition, *Sphingobacterium sp. JCR5* was found to also degrade estrone, estradiol and estriol.

2.7.2.3 Microbial communities in sediment

In general, electron acceptor state plays the important role to regulate structure and diversity of sediment microbial communities (Song et al., 2008).

Xingqing *et al* (2008) found several of microorganisms presented in lake sediment from Taihu. *Bacillas*, *Bacterium*, *Brevibacillus*, *Exiguobacterium*, γ-*Proteobacterium*, *Acinetobacter sp* and some uncultured or unidentified bacteria were predominantly found in the lake sediment. Additionally, the redox potential (Eh) was found in the range of -18 to -130 mV which implied that the redox state of this lake sediment was under sulfate-reducing or methanogenic conditions

Rajendarn *et al* (1996) expressed that the aerobic prokaryotes and eukaryotes were mainly found in surface sediment of Etauchi Bay, Hiroshima Bay, and Kajima lake but the gram-positive bacteria and anaerobic bacteria was dominant in surface sediment Osaka Bay.

Castin *et al* (2009) studied the microbial community diversity of sediment under finfish cage in tropical marine ecosystem, They found that predominant

microorganism were related to δ-*Proteobacteria* which the sequences were belonged to strictly anaerobic genera and sulfate-reducing (*Desulfovibrio*, *Desulfobacter*, *Desulfococcus*, *Desulfonema*, etc) and sulfur-reducing bacteria (*Desulfuromanas*).

2.8 Metabolites of steroid hormones

Metabolites of MT have been found in urine and feces of human and animals. Review of current literature shows that the main metabolites of MT were 17α-methyl-5α-androstan-3α, 17β-diol and 17α-methyl-5 β-androstan-3α, 17β-diol (Figure 2.4) which are more polar than MT (Rongone and Segaloff, 1962; Mosbach *et al.*, 1968; Shinohara *et al.*, 2000 and Williams *et al.*, 2000). It is possible that the degradation pathway of MT by microorganisms in the environment may resemble that of estrogens and testosterone.

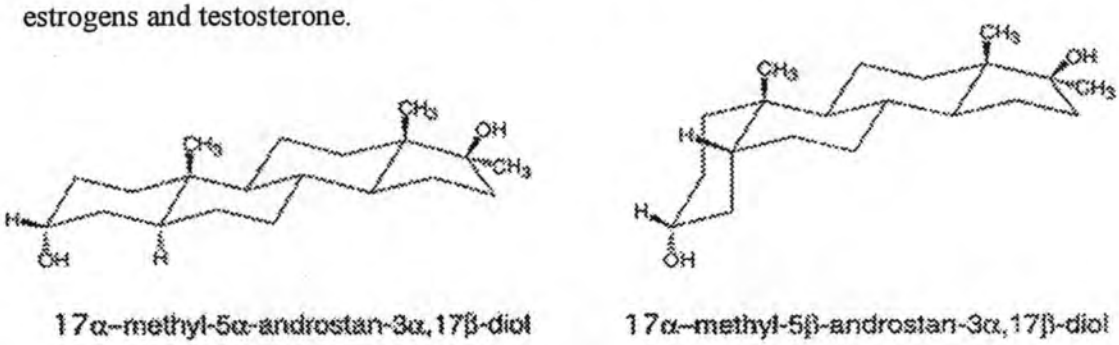


Figure 2.4: Structures of metabolites of MT (Shinohara *et al.*, 2000)

Estradiol is oxidized to estrone as shown in Figure 2.5 (Ternes *et al.*, 1999; Fujii *et al.*, 2002; Shi *et al*, 2004; Weber *et al.*, 2005; Yu *et al.*, 2007).

Figure 2.5: Oxidation of estradiol

Using the supernatant of mixed liquor of activated sludge, Lee and Liu (2002) found that estradiol was oxidized to estrone producing an unknown metabolite as shown in Figure 2.6. However, in the early stage of biodegradation, the unknown intermediate metabolite (X1) was observed. The labile metabolite X1 was identified

by GC-MS as 5-hydroxy-15-methyl-13-oxatetracyclo [8.7.0.0<2,7>.0.<11,15>-heptadeca-2(7),3,5-trien-14-one which was a lactone structure. From the metabolic pathway, it was suggested that the initial degradation of estradiol occurred at the D ring (Lee and Liu, 2002).

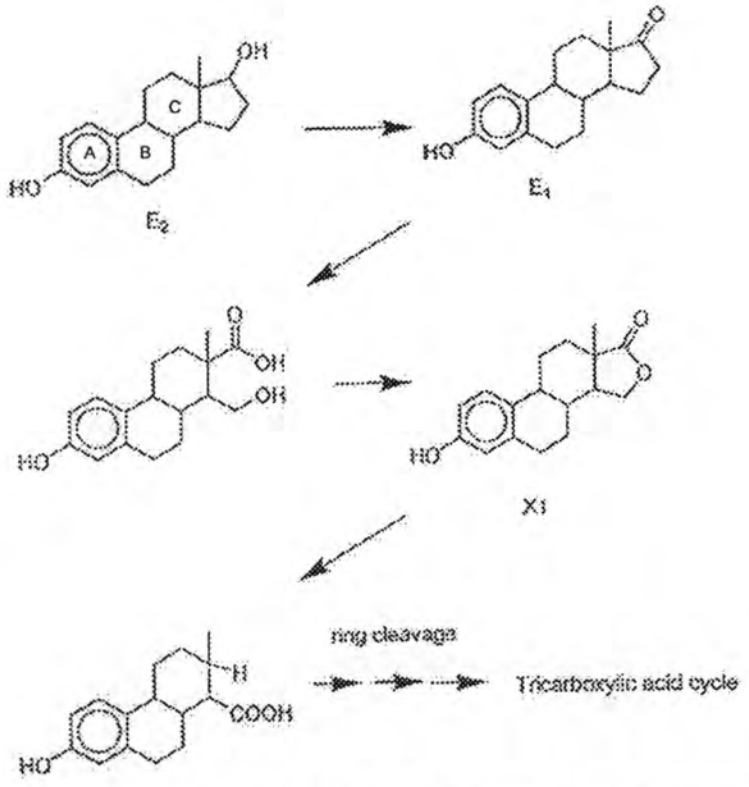


Figure 2.6: Proposed metabolic pathway of estradiol by sewage bacteria (E1= estrone, E2 = estradiol) (Lee and Liu, 2002)

With 3-chlorocatechol which is an inhibitor of meta-cleavage, Ogura *et al* (2004) found that strain ED8 (*Sphingobium herbicidovorans*) degraded estradiol to estrone with five metabolites (I-V) found in neutral extraction and one metabolite (VI) found in acid extraction. Without 3-chlorocatechol, no metabolites was observed except for estrone. The pathway of estradiol biodegradation by strain ED8 with/without 3-chlorocatechol is shown in Figure 2.7. From the GC-MS analysis, metabolite I had spectrum pattern similar to 6α-OH-estradiol but with a different retention time as compared to 6α-OH-estradiol, meaning that metabolite I had the same structure as 6α-OH-estradiol but was not 6α-OH-estradiol. For metabolite II, the spectrum pattern was similar to 7-keto-estrone but the position of keto-group was

unknown. The spectrum pattern of metabolite III was similar to 6-keto-estradiol but the position of keto-group was unsure. For metabolite IV and V, the spectrum patterns were 4-OH-estrone and 4-OH-estradiol. The metabolites found in Ogura *et al.* (2004) study indicated that initial degradation of estradiol occurred at the A ring (hydroxylation at C-4). The difference between the results of Ogura *et al* (2004) and Lee and Liu (2002) may be due to difference in activity of microorganisms in the degradation of estradiol and the use of a meta-cleavage inhibitor.

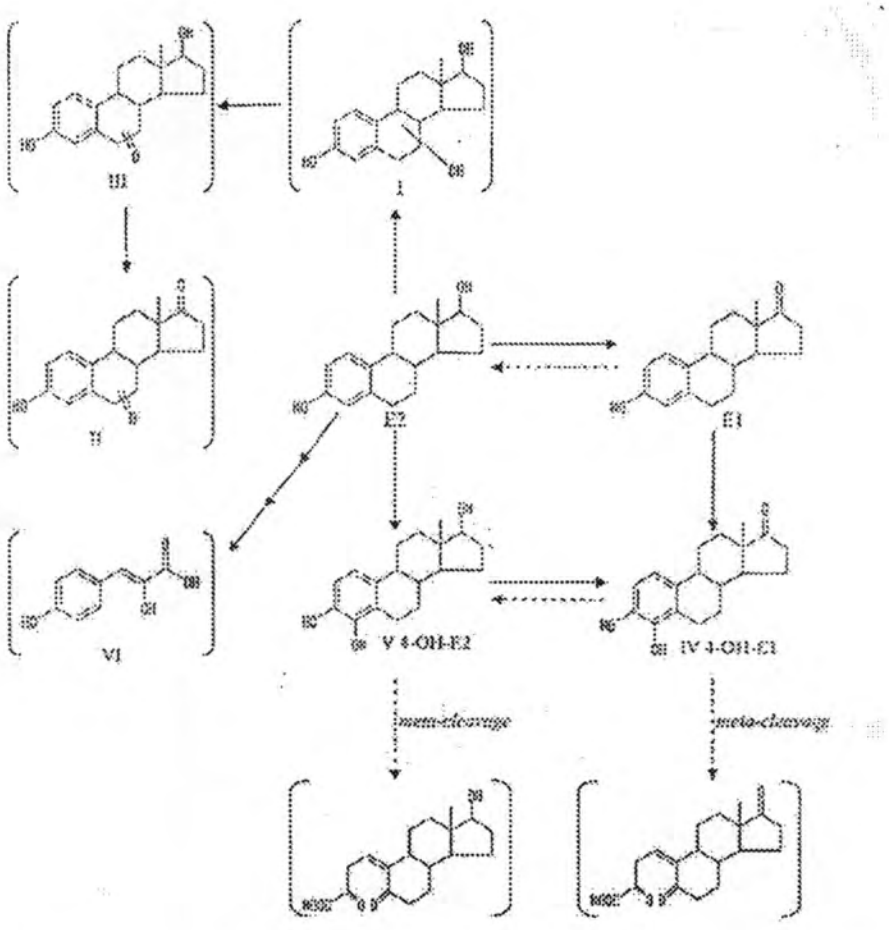


Figure 2.7: Proposed metabolic pathway of estradiol by strain ED8 (E1= estrone, E2 = estradiol) (Ogura *et al*, 2004)

2.9 Androgenicity

Normally, hormones in endocrine system bind to specific membrane receptors and send the response to the target organism for synthesis of the specific protein or

development of tissue (Leusch *et al.*, 2006). For example, testosterone, a natural androgen hormone produced in human and animals, can bind to androgen receptor in target cells and regulate the development of their secondary sex characteristic. The chemicals released by anthropogenic activities including biotransformed products which cause abnormal development of sex organ and have negative impact on the reproductive system are often referred to as endocrine-disrupting compounds (EDCs) (Purdom *et al.*, 1994; Damstra *et al.*, 2002; O'Connor and Chapin, 2003 and Matthiessen, 2003).

In this part, the literature review emphasized on the chemicals which act as androgen hormone can bind to androgen receptor (AR) and contribute to an increase in incidences of male reproductive abnormalities (Kelce and Wilson, 1997). In vitro bioassays can provide an assessment of the overall biological activity of these chemicals in an environmental sample, making them ideal for rapid and large-scale screening. The results from in vitro bioassays are typically presented in term of androgenic potency. The yeast androgen screen (YAS) (Sohoni and Sumpter, 1998) and two hybrid yeast assays (Lee *et al.*, 2003) are the receptor-binding assays available for the analysis of androgenic potency of single compounds or complex mixtures.

Lee *et al.* (2003) developed the novel yeast two-hybrid protein interaction for detection of androgenic and antiandrogenic compounds. A yeast strain, ARhLBD-ASC1, was constructed by co-transformation of yeast cells containing a lacZ reporter plasmid with two vectors expressing each of LexA fused hinge–ligand binding domain (hLBD) of androgen receptor (AR) and B42 fused ASC-1 that interacts with the AR-hLBD in an androgen-dependent manner. This bioassay can be carried out with the simple X-gal staining on microtiter plates. However, this bioassay has a lower sensitivity than other mammalian cell bioassay with AR and androgen responsive reporter gene performing in some cell lines such as DU-145, COS, CHO, CV-1,MCF7 and HepG2 (Berrevoets *et al.*, 1993; Miyamoto *et al.*, 1998; ; Maness *et al.*, 1998; Kemppainen *et al.*, 1999; Lobaccaro *et al.*, 1999; and Vinggaard *et al.*, 1999). The EC_{50} of testosterone and DHT measured by overall mammalian cell bioassay were in range of 0.002-20 and 0.04-3 nM, respectively (Lee *et al.*, 2003).

Sohoni and Sumpter (1998) determined the androgenic activity of dihydrotestostreone (DHT) and testosterone (T) and found that the androgenic

activities of both compounds were similar. Moreover, they found that the androgenic activity of diethylstilboestrol (DES) was about 80,000 times less than that of DHT. Other chemical such as vinclozolin, 1,1-dichloro-2,2-bis(p-chorophenyl)ethylene (p,p'-DDE) and 4-Nonylphenol (4-NP) also express some androgenic activity, but their potent were weaker than that of DES. However, not much data are available on the androgenic activities of xenobiotics (Sohoni and Sumpter, 1998).

Gaido *et al.* (1997) determined the activity of testosterone and DHT by using the yeast-based, human androgen steroid hormone, receptor gene transcription assay. They found testosterone and DHT had similar activity with potency ratios of about 1.35 and 1.00, respectively, based on the activity of DHT.

Rijk *et al.* (2008) used the yeast androgen bioassay with green fluorescent protein (yEGFP) to determine the relative androgenic potency (RAP) of several hormone compounds. The RAP is the ratio of the EC_{50} of 17β-testosterone and the EC_{50} of other hormones. The result showed that the RAP of 17α-testosterone, 19-nortestosterone, 17β-boldenone and 1-testosterone were 0.0063, 1.6, 0.18, and 1.9, respectively. This suggested that 17α-testosterone showed the lowest response to human androgen receptor (hAR) in comparison with other androgen hormones based on the 17β-testosterone.

Urbatzka *et al.* (2007) studied the androgenicity in the river Lambro, Italy, which was contaminated various endocrine disruptor compounds such as pesticides (Vigano *et al.*, 1999), trace metals (Pettine *et al.*, 1996), and organochlorines (Vigano *et al.*, 2000). The androgenicity was detected by YAS measured in terms of methyldihydrotestosterone (MDHT) equivalent, an androgen hormone They found androgenic in term of MDHT equivalent in some fractions of the water were in range of 130.05 ± 38.58 ng/L – 207.51 ± 47.34 ng/L and in sediments in the range of 22.99 ± 2.12 µg/kg – 40.01 ± 14.23 µg/kg of sediment but no androgenicity was detected in the total extraction.

Thomas *et al.* (2002) found that the androgenic potentials in rivers and estuaries in United Kingdom were as high as 10 ng/L DHT-equivalent, in effluents from sewage treatment plants were as high as 635 ng/L DHT-equivalent, and in sediments were as high as 15.3 µg/kg DHT-equivalent.

Leusch *et al.* (2006) determined the relative binding affinity of androgen hormones with an androgen receptor isolated from trout brain and found that MT has

a relative binding affinity of about 0.025 compared to testosterone. This value was lower than the relative binding affinity of androstenedione and androstadienedione about 4.13 and 2.03, respectively.

Bandeli *et al.* (2006) studied the relative potency of androgen hormones by using in vivo bioassay. They found that potency of MT was about two orders of magnitude more than that of androstenedione and androstadienedione which are natural androgen hormones.

2.10 Summary

From the literature review, it is highly probable that the uneaten and unmetabolized MT-impregnated food accumulate in the sediment and water of masculinization pond environment. Discharge of contaminated sediments and water into receiving waters or onto land disposal sites are the main pathways of distribution of MT in the environment. Residual MT in the water or sediments from the masculinization process can impact animals (aquatic lives and invertebrates), humans and the ecosystem. MT, an endocrine disrupting compound, can interfere with the normal functions of reproductive system and can cause an imbalance in the sex of wildlife. MT concentrations as low as nanogram per liter level were found to induce abnormality in wildlife. A few studies found the occurrence of MT in sediment and water of masculinization ponds. Not much is known about the fate of MT. There are no studies on the biodegradation of MT in sediments or in water under various environmental conditions. Understanding the biodegradability of MT will provide information on the persistency of MT in the environment and the extent at which MT can impact the environment and humans.