Microbial Degradation of Steroid Hormones in the Environment and Technical Systems

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Abstract

Steroid hormones are naturally produced by human and animals. Most important steroids are 17β-estradiol (E2), estrone (E1) (both estrogens), testosterone as well as the synthetic ethinylestradiol (EE2). All substances consist of four carbon rings what makes them stable in the environment. Steroid hormones are secreted in urine and mainly enter the environment by waste water treatment plant (WWTP) effluents. The problem of steroid hormones is that they are endocrine disruptors and can affect aquatic organisms such as fish. Effects like feminism of male fish have already been observed near WWTP effluents. The most important process to remove the steroids is the microbial degradation. Sorption and to minor extent photo degradation can also play a role in the removal of these hormones. The degradation rates of E2, E1 and EE2 have been studied widely. It was found that E2 is oxidised to E1 in the first step. The half-live of this step is around 4 to 12 hours in aerobic water and soil. However, this step does not significantly reduce the estrogenic potential. Further degradation of E1 needs the cleavage of one ring. Therefore, half-lives of E1 are significantly higher and observed E1 concentrations are normally higher than E2 concentrations. The degradation of E1 shows a linear relationship with the removal of the estrogenic potential. The structure of EE2 is analogue to E2 but there is an ethinyl group at one hydroxyl group containing C-atom. This group is normally vulnerable to microbial attack. A cleavage of this ring is therefore difficult what makes EE2 much more recalcitrant in the environment. Thus, EE2 has a big impact on the estrogenic potential although the secreted amount is much smaller than that of E2 or E1. The degradation rates of steroid hormones in anaerobic sediments are much smaller. Half-lives of anaerobic E2 degradation are in the range of tens of days. E1 and EE2 can often not be degraded under anaerobic conditions. Therefore these substances accumulate in these environments. In the WWTP the half-lives of E2 are in the range of a few minutes under aerobic and denitrifying conditions whereas E1 shows half-lives of up to one hour in the denitrification tank. E2 and E1 are thought to be degraded metabolically, i.e. for the bacteria to gain energy. EE2 is degraded within 2 to 4 hours under aerobic conditions in the WWTP but is quite persistent under denitrifying conditions. EE2 is thought to be degraded cometabolically, i.e. incidentally without an energy gain for the organism, by an enzyme of nitrifying bacteria, but this is not fully understood yet. The much smaller half-lives in the WWTP compared with the natural systems are due to the much higher bacteria density. Nevertheless, it is still controversial which part of the WWTP is most efficient concerning steroid removal. The metabolic degradation pathway of E2 and E1 is still not clear. The metabolic initial cleavage of the aromatic ring of E1 was proposed forty years ago but the cleavage of the five-ring as initial step was recently suggested, too. On the basis of electron density comparisons it was assumed that EE2 is firstly cleaved at the aromatic ring as well. On the other hand, the degradation pathway of testosterone – the so called 9,10-seco pathway - is well described and starts with the cleavage of a ring in the middle. To improve the efficiency of WWTP concerning removal of steroid hormones an increased sludge retention time (SRT) and hydraulic retention time (HRT) as well as the introduction of the most efficient bacteria community are of most importance.

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1. Introduction

1.1. What is the problem?

Steroid hormones are naturally occurring organic substances which are mainly produced by human and animals. They are human sexual hormones. Estrogens are the most important female sexual hormones which are produced in female ovarian. The most abundant estrogens are 17β-estradiol (E2) and estrone (E1). Male sexual hormones are called androgens and are produced in the testes. The most important androgen is the testosterone (Fahrbach 2006). These hormones are excreted by the urine of human or animal body (Pauwels et al. 2008), normally in a conjugated form as soluble and inactive glucorinides. This conjugated form is then microbial cleaved in the environment or in the waste water treatment plant (WWTP) (Jurgens et al. 2002). Although the hormones are naturally produced there is an increasing concern about its effects in the environment as they act as endocrine disruptors. Since population grows and more people live in large cities all excreted hormones are entering the environment at one point: the effluent of the WWTP. In addition, ethinylestradiol (EE2), the major compound of the contraceptive pill (which is also excreted by the urine) enhances the concentration of hormone-like substances in the WWTP effluent. However, only 1% of all excreted estrogens is EE2 whereas 80% are estrogens produced by women (De Mes et al. 2005). Concentrations of estrogens in the environment are mostly lower than five ng/L whereas concentrations in the WWTP effluent can exceed fifty ng/L (table 1). Due to the higher persistence of EE2 in the WWTP (see section 1.2), the concentrations of the synthetic estrogen in the environment is analogue to concentrations of the natural estrogens despite it is excreted in a much smaller amount. However, concentrations in WWTP differ largely depending on the amount of substances entering the plant and how efficient the treatment is. Concentrations in the river depend on how far the river sample was taken below a WWTP effluent. Galli & Braun (2008) claim that highest river concentrations are found where effluents of low-efficient WWTP discharge into small rivers. This is especially the case in summer during dry periods when the effluents are not diluted enough (Jurgens et al. 2002). Not shown in table 1 is that in the same systems concentrations of E2 are mostly much smaller than E1 concentrations. This is due to the fact that E1 is more persistent concerning microbial degradation (see section 1.2). With respect to testosterone concentrations in WWTP the database is not clear since only a few studies were done. Not only WWTP but also runoff from agriculture production and manure applications contribute to the release of steroid hormones in the environment (Fahrbach 2006). There, testosterone plays an important role (Lee et al. 2003). That means that soil can also be affected by the sexual hormones.

Table 1: Concentration range (ng/L) of E2, E1 and EE2 found in WWTP effluents and rivers.

	WWTP effluent	River
E2	n.d[1].-64	n.d.-5.5
E1	n.d.-47	n.d.-3.4
EE2	n.d.-42	<5

1 n.d. not detected

References: Williams et al. (2003), Ternes et al. (1999b), Stumpf (1996), Belfroid et al. (1999)

As mentioned above, steroid hormones are endocrine disruptors. This means that if they exceed a certain level in the environment it can lead to a misbalance of the endocrine system in animals. Effects on fish have often been reported. These include feminism of male fish, decreased growth of the testes and vitellogenin (an egg yolk precursor protein) production in male fish which results in reduced reproduction. Panter et al. (1998) for example found that fathead minnows show these effects if they live in water which was spiked with steroid hormones in concentrations which can be observed in the environment (table 1). Analogue to this, Purdom (1994) concludes that EE2 concentrations in the range of 1-10 ng/L (i.e. concentrations that have been observed in rivers) could induce vitellogenin production in male rainbow trout. Thus, the fact that fishes show endocrine disruptions in concentrations observed in the environment means that efforts must be taken to diminish this problem.
In addition to the natural occurring steroid hormones and their synthetic imitations, so called xeno-estrogens can also act as endocrine disruptors (Galli & Braun 2008). Industrially produced nonylphenol and bisphenol A but also metabolites of pesticides (e.g. of DDT) have an effect on aquatic organisms due to interactions with the estrogen-receptor (although they are no steroids). It was shown that after the nonylphenol ban in Switzerland in 2006 the endocrine activity in rivers near highly industrial areas decreased (Galli & Braun 2008). However, investigations showed that the contribution of endocrine activity of nonylphenol is low in comparison with steroid hormones and that the activity of bisphenol A can nearly be neglected (Sumpter & Johnson 2005). It was also shown that EE2 has a 20 times higher estrogenicity (concerning vitellogenin induction in rainbow trout) than E2 whereas the estrogen activity of E1 is only one third compared with E2 (Sumpter & Johnson 2005). In the next section it is shown why steroid hormones are quite stable in the environment and through which processes they can be degraded.

1.2. Structure of steroid hormones, stability and degradation processes

Figure 1 shows the molecular structures of the most important steroid hormones and their derivates. They are all built from cholesterol. The A ring shows phenolic properties whereas the other rings do not contain any double bonds. Due to the four linked rings the steroid skeleton is very stable. Estrogens and androgens contain three quaternary C-atoms: the C-5, C-10 and C-13 (Figure 1). Not many sites are available where a simple oxidation of a C-atom can take place as the rings are hardly substituted. Thus, the energy gain by the degradation of steroids is very low. In the case of EE2, degradation is even more difficult since the introduction of the ethinyl group makes the ring very stable against oxidation at the position 17 (de Mes et al. (2005) and Fahrbach (2006)).
In the environment steroid hormones can be removed differently. This includes sorption, photolytic degradation as well as microbial degradation. There is a lot of literature dealing with sorption (e.g. Casey et al. (2003), Casey et al. (2004), Cirja et al. (2007), Lee et al. (2003)), but less about photolytic degradation was reported (e.g. Liu et al. (2003), Zuo et al. (2006)). Most studies are about microbial degradation of steroid hormones. In WWTP, the natural processes sorption and degradation are the two processes that play the major role. In addition to this, different technical elimination processes as tertiary treatment are tested to date. Amongst others are ozone oxidation (Baig et al. 2008), inverse osmosis (Khan et al. 2004), ultrasound (Suri et al. 2008), nanofiltration (Weber et al. 2004) and photo-fenton reactions (Zhao et al. 2008). This report solely deals with microbial degradation in the environment and in the WWTP. It is scientifically accepted that the first step of the degradation of E2 is the oxidation of the C-17 to a keto-group, hence the formation of E1.

This step is suggested to be a rather fast step as this is a simple oxidation which can be done by ubiquitous enzymes. The further mineralisation and the factors favoring the degradation are still controversial. The degradation of E1 must be induced by a ring cleavage what is supposed to be a slow step. That is why E1 concentrations in the WWTP effluent are normally higher than E2 concentrations (see section 1.1) although E2 is excreted in a much larger amount. Because of the different first order degradation rates of E2 and E1, one often finds the typical concentration profile shown in figure 2 for the degradation of E2. E2 is removed fast, accompanied with the formation of E1 which is then degraded much slower.

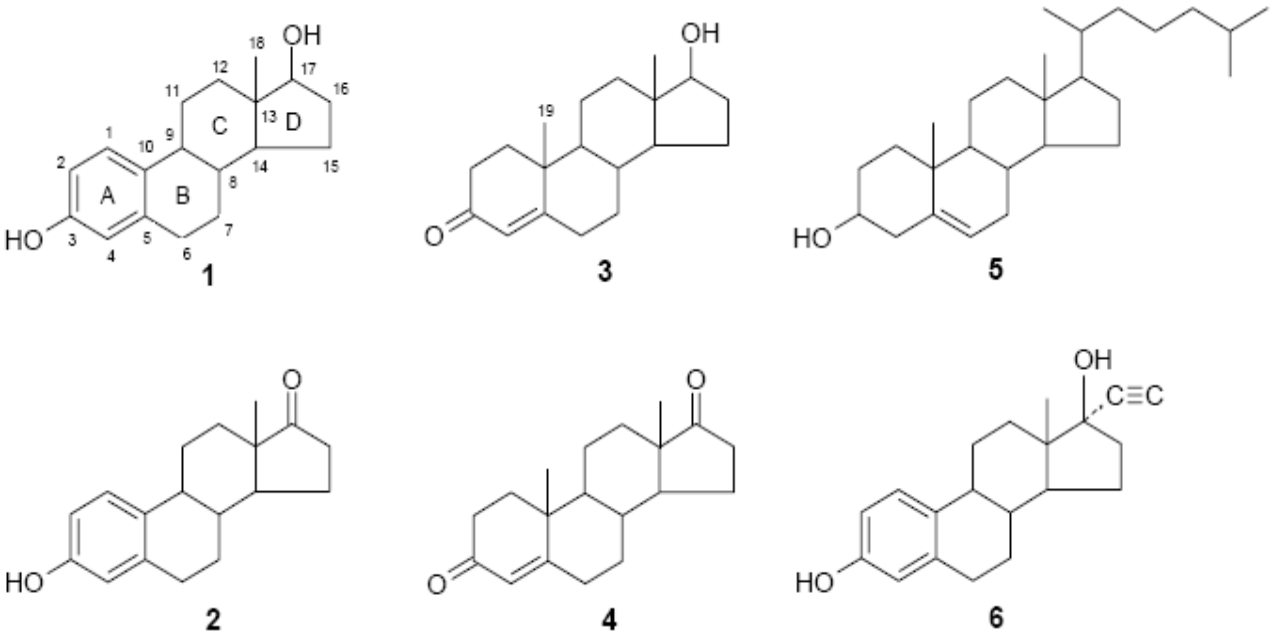

Figure 1: Molecular structures of the steroid hormones estradiol (E2) 1, estrone (E1) 2, testosterone (T) 3, 4-androstene-3,17-dione 4, their precursor cholesterol 5 and the main substance of the contraceptive pill ethinyl estradiol (EE2) 6 (Fahrbach 2006).

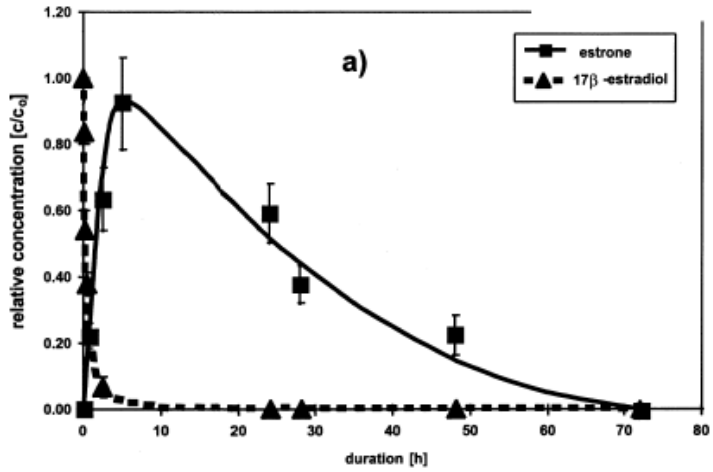

Figure 2: Typical profile for the degradation of E2. It starts with a fast degradation of E2 and the simultaneous built up of E1 followed by a much slower degradation of E1. Data taken from Ternes *et al.* (1999a)

The aim of this report is to explain differences between the microbial degradation of steroid hormones in water, soil and sediment and to compare typical natural degradation rates with such in WWTP. This is illustrated in section 2. In section 3, different proposed degradation pathways are compared and it is discussed which bacteria types could be most relevant for the steroid degradation in the WWTP. This section goes more into the molecular detail of the steroid degradation and the differences between energy gaining metabolism and cometabolism. With regard to the adaption of specific bacteria culture it is important to know how a degradation pathway looks like and how different bacteria types deal with these substances. As it is seen later, this is still not a very well understood issue. Section 4 gives a conclusion of the review and recommendations for further studies.

2. Degradation efficiency in the environment and in the WWTP

2.1. Degradation in the water

Jurgens et al. (2002) did a broad study about microbial degradation of E2 and EE2 in English rivers. It was shown that aerobic degradation in urban and industrially affected rivers was much faster in summer than in winter. Shortest half-lives from around 4 to 5 hours were found for the degradation of E2 in summer at 20°C (cf. longest half-lives around 40 hours). The same rates were found for the degradation of E1. This is in contrast to the theory that oxidation of E2 to E1 is a fast step whereas the ring cleavage of E1 is a slow process (section 1.2). However, the fact that E1 concentrations in the environment are normally higher than E2 concentrations means that this experiment did not reflect the reality.
Half-lives in winter and spring were 2.5 to 8.5 times higher than in summer (also tested at 20°C). Explanations could be higher prior temperatures in summer or higher nutrient concentrations due to less dilution in summer. This means that microorganisms that were exposed to the test system had a higher initial activity. Interestingly the river in the agriculture area did not show such a correlation. It was argued that the microorganism concentration in this river was always much smaller than in the other samples. Consequently half-lives of E2 degradation could rise up to 9 days. Another interesting feature was that E2 removal with an initial concentration of 100 ng/L was faster than with 100 µg/L. The same effect was shown by Ternes et al. (1999a). When only 20 ng/L was spiked, the degradation rate was similar as with 100 ng/L. To Jurgens et al. (2002), the finding that E2 can be degraded at environmental concentrations (with similar half-lives as with high concentrations) means that degradation occurs without the multiplication of E2 degrading bacteria. Thus, E2 degrading bacteria did not need to adapt prior the degradation. It was also shown that degradation of E2 leaded to the buildup of E1 in a first step which was then further mineralised to CO_2 (without the knowledge of the intermediates). The reaction of E2 to E1 did not change the estrogenic activity a lot. However, after the degradation of E1 hardly any estrogenic activity was observed. The comparison of the degradation efficiencies of EE2 and E2 showed that EE2 had a much longer half-life up to 17 days (compared with 1.2 days for E2) (Jurgens et al. 2002). Hence, EE2 was more recalcitrant in the water. This is due to the fact that EE2 has an ethinyl group at position 17 which hinders the oxidation at this C-atom (section 1.2) and stresses that although EE2 concentrations in natural water are much smaller than E2 concentrations (de Mes et al. 2005), EE2 can have a big impact on the environment due to its stability. An additional way how EE2 can be degraded is by photolysis. Jurgens et al. (2002) was able to find a half-life for the photolytic degradation of E2 and EE2 from around 10 days (at 12 hours sunshine per day) which means that EE2 removal through photolysis could be significant at least at places where the sun is often shining.

A study investigating the degradation of endocrine disrupting chemicals in seawater found that E2 and EE2 (initial concentrations 5 µg/L) hardly decreased within the first 28 days followed by a rapid decline in the next 14 days (Ying & Kookana 2003). However, in comparison with the study of the English rivers (Jurgens et al. 2002) degradation of EE2 was as fast as the one of E2. There is no explanation of this differences and nothing is said about the origin of the lag phase in the marine system. Possibly a bacteria culture could adapt to the new conditions with the steroids. Nevertheless, it is questionable if in real seawater (where estrogen concentrations do not exceed 50 ng/L) an estrogen degrading bacteria culture like in the experiment can really adapt.

2.2. Degradation in the soil

In soils sorption is a second process besides biodegradation which can play a role and it is often difficult to determine which process is responsible for the decrease of the substance concentration. To distinguish these removal processes experiments are mostly carried out with an autoclaved control sample that is treated like the non-sterile sample.
Colucci et al. (2001) found that the first step of the degradation of E2 (the oxidation to E1) was fast in the non-sterile sample but also occurred in the autoclaved sample but in a much slower extent. The fact that oxidation in the autoclaved sample occurred at all leaded to the conclusion that the oxidation can also take place abiotical. Nevertheless, one should account for that sterilising soil is not an easy task at all. Thus, although the soil was sterilised twice, some bacteria could still be active and responsible for the slow degradation in the autoclaved soil. In contrast, the further degradation of E1 did not happen in the autoclaved soil indicating that these steps needed the presence of microorganisms. However, only in the loam soil but not in the sandy loam or silt loam soil respectively further degradation of E1 occurred. Colucci et al. (2001) argued that in addition to this, it is not clear if due to autoclaving changes in the soil property happened which could affect sorption. This means that sorption could be possible to appear in the soil but was made inoperative for it because of the autoclaving. The dissipation of E1 was done without any lag phase (Colucci et al. 2001). This means that microorganisms did not need to adapt in order to degrade the steroids. It was concluded that estrogens and related substances have been ubiquitous in the environment for a long time because they act as regulatory chemicals in animals. Hence, bacteria have evolved enzymes to degrade these chemicals. In the study a half-life of 0.2-0.5 days and 0.6-1.7 days was found for the dissipation of E2 and E1 respectively (when the soil was incubated with 1 mg/kg substrate at 30°C). The differences within one substance were due to the different soils. E1 is around three times more persistent than E2 what is also supported by Xuan et al. (2008) (they found half-lives of 0.92 and 2.7 for E2 and E1 respectively in 20% non-sterile soil). This is equivalent with the theory that E1 is more persistent because one ring needs to be cleaved (section 1.2) and is also compatible with the fact that E1 is found in larger concentrations than E2 (section 1.1).
In contrast, a half-life of 2.1 days was found for the dissipation of EE2 in loam soil under the same soil conditions (Colucci & Topp 2001). This is 2.3 times and 7.2 times longer than the half-lives of E1 and E2 in the loam soil (Colucci et al. 2001). Half-lives increased by a factor of 1.5 if the initial concentration of EE2 was ten times higher (cf. section 2.1). Together with the loss of EE2, the estrogenicity decreased i.e. no other endocrine disruptive substance was formed. The EE2 was more persistent in drier soils and at lower temperatures. The same pattern was shown by Xuan et al. (2008) for E2. The half-life at 20°C was 2.1 times shorter than at 10°C what fits exactly with the rule of Arrhenius. With all these data it can be concluded that EE2 was actually degraded biological. At first, the autoclaved control soil did

not show an EE2 decrease, at second the removal was much slower in an oxygen free atmosphere and at third the removal increased at higher temperatures following the rule of Arrhenius. However, the pathway of the degradation could not be found out. Colucci & Topp (2001) argue that the initial step of the degradation of EE2 is probably not similar to that of the degradation of E2 as they tried to feed EE2 to bacteria which have been living on E2 as sole carbon source what did not work (see section 3.1 for proposed pathways). Arcand-Hoy et al. (1998) showed that the first step of EE2 degradation in human is the hydroxylation of the C-2 at Ring A. However, it is not yet clear if the same step is done by bacteria in the environment.

2.3. Degradation in the sediment

As sediments are often anaerobic (no oxygen available) studies in sediments can be used to assess the differences of steroid hormone degradation under aerobic and anaerobic conditions. Sorption to particles like in the soil can also play an important role in sediments. Jurgens et al. (2002) investigated river sediments and found that degradation of E2 and E1 was faster in the aerobic than in the anaerobic sediment. Nevertheless, for E2 they also found quite low half-lives from around 0.5 days in the anaerobic sediment (compared with 0.1 days in the aerobic sediment). These values are in the same range like the aerobic degradation in the water (see section 2.1). Because half-lives of E1 in the anaerobic sediment were around 13 days, it was concluded that this substance would accumulate. In aerobic marine sediments a half-life of 4.4 days was found for E2 degradation, while in the anaerobic sediment the E2 concentration was only halved within 70 days (Ying & Kookana 2003). It was assumed that sulfate was the main electron acceptor for the oxidation of E2 to E1.

Czajka & Londry (2006) analysed the degradation of EE2, E2 and E1 in lake sediments under anaerobic conditions with different alternative electron acceptors. E2 could always be degraded but very slowly and only to E1. Half-lives were found to be around 21 days under nitrate reducing conditions, 6 days under iron reducing, 9 days under sulfate reducing conditions and 15 days with methanogenesis. That means that degradation was much less efficient than in the anaerobic river sediments. The large differences were due probably to differences in the sediment composition. The sterile control showed significantly smaller decrease what is evidence that the reaction was microbial induced. The half-lives also show that the transformation did not correlate with the redox cascade. This was expected as it was assumed that the oxidation requires an NADH-dependent hydroxyl steroid dehydrogenase (Czajka & Londry 2006). Therefore, E2 oxidation under different anaerobic conditions rather runs with the help of different enzymes. As the conversion was only to E1 and not further the total estrogenicity did not decrease a lot. The formed E1 could react back to E2 so that equilibrium was reached where (depending on the anaerobic conditions) 12-50% of the total estrogens were in the oxidised form of E1. It was found that EE2 could not be removed under any anaerobic conditions, either in the river sediment (Jurgens et al. 2002), the lake sediment (Czajka & Londry 2006) nor the marine sediment (Ying & Kookana 2003). Czajka & Londry (2006) claim that estrogens would accumulate in anaerobic sediments and therefore affect bottom feeding invertebrates and in addition to this can become a source of endocrine disruptive substances if such a system is disturbed.

2.4. Comparison of degradation rates in the natural system

Table 2 shows half-lives found in the literature for the three described natural systems. It is not easy to draw conclusions as experiments were mostly done in different ways. Despite of this, some general points can be considered. It is visible that half-lives of E2 and E1 in aerobic water are in the same range whereas in aerobic soil and sediment degradation of E2 is significantly faster than E1 degradation. As mentioned in section 2.1, the facts that degradation rates of E2 are similar to that of E1 (like it was observed in rivers) do not reflect the reality. In addition, the removal of EE2 is much slower than that of E2 and E1 because of the ethinyl group that makes it more recalcitrant (see section 1.2). Another important point is that degradation under anaerobic conditions is mostly slower than under aerobic conditions or degradation is even impossible. It is not clear why degradation of E2 in the anaerobic river sediment is much faster than in the anaerobic marine and lake sediment respectively. One reason could be that the anaerobic river sediment still contained a tiny amount of oxygen as it is difficult to create a real anaerobic environment.

Table 2: Summary: half-lives of E2, E1 and EE2 degradation in natural systems.

	Conditions:	Half-life E2/d	Half-life E1/d	Half-life EE2/d	Reference:
Water:	aerobic	0.2-1.7[b]	0.1-1.8[b]	17	Jurgens et al. (2002)
Soil:	aerobic	0.17[c]-0.5	0.6-1.7	2.1-3.2	Colucci & Topp (2001) and Colucci et al. (2001)
Sediment:	aerobic (river)	0.1	0.42	-	Jurgens et al. (2002)
	aerobic (marine)	4.4	-	>20	Ying & Kookana (2003)
	anaerobic (river)	0.4-0.7	11.5-14.3	-	Jurgens et al. (2002)
	anaerobic (marine)	70	-	n.d.[e]	Ying & Kookana (2003)
	anaerobic[a] (lake)	6.0-21.0	-[d]	n.d.[e]	Czajka & Londry (2006)

a: NO_3^- reduction, Fe^{3+} reduction, SO_4^{2-} reduction, methanogenesis
b: samples taken in summer in industrially affected rivers
c: Xuan *et al.* (2008)
d: back reaction to E2
e: not degraded

2.5. Degradation in the WWTP

The degradation of steroid hormones in the WWTP has widely been investigated as most of these endocrine disruption substances enter the plant before they are released to the environment. If it is managed to fully degrade the hormones before they enter the environment, effects on fish and other animals as mentioned in section 1.1 can be minimised.

Differences between facility types

There is a big variation in removal efficiencies depending on the running properties and the size of the WWTP. In a big German sewage treatment plant with activated sludge it was found that 98% of E2 and E1 as well as more than 90% of EE2 was removed during the process (Andersen et al. 2003). Only 5% was sorbed onto the sewage sludge. In addition, Ternes et al. (1999a) excludes adsorption on activated sludge since in there experiment the whole estrogen amount was almost found either unchanged or as metabolites. On the other hand, a plant with membrane bioreactor (MBR) sorbed 80% of the total estrogens whereas only 1% was really mineralised (Cirja et al. 2007).

Metabolic and cometabolic degradation in the different tanks

Andersen et al. (2003) showed that the degradation of E2 and E1 mainly occurred in the denitrifying and nitrifying tank while EE2 was only removed in the nitrifying tank. Fahrbach (2006) was the first to isolate two denitrifying bacteria strains from the active sludge of a municipal WWTP which were able to live on estrogens as sole energy and carbon source. Such bacteria could be part of the community in the denitrification tank which leads to the rather fast degradation. However, there are many other denitrifying bacteria in the tank that it is not possible to say which strain is most abundant and most efficient in degrading steroids. It is also not clear how the strains found by Fahrbach (2006) react on much smaller concentrations than induced in the experiment. In addition to this, bacteria which are degrading steroids without gaining energy but rather incidentally (so called cometabolically, see section 3.2) should also be taken into account. Like in the water and soil the synthetic EE2 is much more persistent in the WWTP because of the ethinyl group at the C-17 which hinders the oxidation of the hydroxyl group at the same site (section 1.2). Not a lot of bacteria are able to degrade EE2 when it is added as sole carbon and energy source. *Sphingobacterium sp. JCR5* (Ren et al. 2007), *Rhodococcus zopfii* and *Rhodococcus equi* (Yoshimoto et al. 2004) were reported recently to be strains which can degrade EE2 metabolically. Here as well, cometabolically active bacteria should be taken into account (see section 3.2). On the other hand, E2 and E1 are thought to be degraded metabolically under aerobic conditions, i.e. to gain energy (de Mes et al. 2005).

Comparison of half-lives between WWTP and the natural system

Due to the different facilities of WWTP, half-lives of the different estrogens are difficult to be specified. Table 3 shows half-lives of the E2, E1 and EE2 degradation in aerobic and denitrifying tanks of WWTP from different experiments. It can be seen that depending on the experimental conditions the half-life of E1 is a factor of two to forty times larger than the one of E2. This means that E1 is more persistent in the WWTP what is again due to the fact that E2 is removed by a simple oxidation whereas for the E1 degradation one ring must be cleaved (see section 1.2). The larger persistence of E1 was also shown in aerobic soils and sediment (see table 2). Therefore effluent concentrations of E1 are larger than that of E2 despite the fact that E2 is secreted in a much larger amount (see section 1.1). The much larger half-life of EE2 in comparison with the natural estrogens (20 to 130 times larger than that of E1) in addition with lower input concentrations of EE2 (see section 1.1) results in similar WWTP effluent as well as similar river concentrations of EE2 as concentrations of natural estrogens. The comparison of aerobic with denitrifying conditions showed that E2 was removed quite efficiently under both conditions. On the other hand, E1 was transformed much more efficiently under aerobic conditions, the half-lives under denitrifying conditions were three to six times larger (table 3, only comparing the half-lives from Joss et al. (2004)). If half-lives of hormone degradation in natural systems (table 2) are compared with the ones from the WWTP (table 3) it is obvious that the degradation in WWTP is much faster. In natural systems minimal E2 and E1 half-lives are in the range of two to four hours and even several days for EE2. On the other hand, in the WWTP half-lives of E2 and E1 are in the range of minutes whereas EE2 concentration is halved within hours. The difference is mainly due to the higher bacteria concentrations in the activated sludge than in the environment. In WWTP the ratio of the degradation rate of E2 to E1 shows a large variation of 2-40 whereas in soil this ratio is around 3. The big variation in WWTP is due to the fact that the running properties of WWTP differ a lot. The half-life of EE2 in the WWTP was 20-130 times larger than the one of E1 which is comparable with the ratio in the water (10-170). However, in soil EE2 was only degraded 2-4 times more slowly than E1.

Table 3: Half-lives of E2, E1 and EE2 degradation in aerobic and denitrifying tanks of WWTP.

Conditions:	Treatment:	Half-life E2/min	Half-life E1/min	Half-life EE2/min	Initial conc./ ng/L[c]	Reference:
aerobic:	Activated sludge	1.3	2.5	336	500	Kjølholt J (2004) in De Mes et al. (2005)
aerobic:	Activated sludge	2.9	6.2	126	500[d]	Joss et al. (2004)
	MBR[a]	1.1	2.3	168	500[d]	Joss et al. (2004)
aerobic:	Activated sludge	2.1	45	n.d[b]	1000	Ternes et al. (1999a)
denitrifying:	Activated sludge	2	72	5940	500	Kjølholt J (2004) in De Mes et al. (2005)
denitrifying:	Activated sludge	2.2	33.3	834	500[d]	Joss et al. (2004)
denitrifying:	MBR[a]	3.6	8.7	336	500[d]	Joss et al. (2004)

a: membrane bio reactor
b: not degraded
c: if initial concentrations were higher, much higher half-lives were found (De Mes et al. 2005)
d: initial concentration of EE2 was 100 ng/L

It is important to note that estrogens mainly enter the WWTP in conjugated forms (see section 1.1). In a first step of the treatment they are cleaved what results in an increase in estrogen concentrations. Adler *et al. (*2001) showed that the conjugated form of estrogen made up for around 50% of the total estrogen concentration in the WWTP influent. The conjugated forms of the estrogens can be quite persistent in the WWTP. Hence, the transformation of the conjugated estrogen to the free estrogen could take much time so that there is not enough time left to subsequently degrade the unconjugated estrogens. However, this issue is often not taken into account in the studies and the importance of conjugated forms is not clear yet.

Influence of WWTP system variables

Sludge experiments with different initial estrogen concentrations showed that degradation is significantly higher at low initial concentrations. The same effect was observed in the water (see section 2.1). Layton et al. (2000) investigated the temperature dependency of the removal of estrogens and came to the conclusion that higher temperatures accelerated the degradation of E2 and E1 whereas EE2 and testosterone were not affected. It was also shown that the removal efficiency of the estrogens can be enhanced with longer hydraulic retention time (HRT) and sludge retention time (SRT) (de Mes et al. 2005) A long HRT means that there is more time for degradation while with a long SRT a specified bacteria culture can adapt (Koh et al. 2008). This is particularly important for slow growing bacteria such as nitrifiers. At longer SRT they have enough time to grow before they are washed out. The smaller half-lives for the degradation of E2 and E1 in a membrane bio reactor (MBR) compared with activated sludge (table 3) is a result of longer sludge SRT and smaller floc size (therefore larger specific surface area) (Joss et al. 2004). The substrate loading, i.e. the COD of the water, also influences the removal efficiency. Joss et al. (2004) assumed that the high COD in the primary effluent inhibits the degradation of E2 and E1 because easily degradable organic matter is used up firstly.

An interesting result is that the degradation of estrogens in activated sludge of a municipal WWTP was quite efficient (84%) whereas the degradation in sludge from an industrial treatment plant hardy occurred (4%) (Layton et al. 2000). This leaded to the conclusion that in the municipal plant an estrogen degrading microbial population adapted since estrogens

have been entering this WWTP for years. In contrast, no estrogens have flown through the industrial plant what did not lead to the adaption of such bacteria. Nevertheless, it was found that testosterone could also be degraded in the industrial plant. The reason of that is unclear. It could be that testosterone-like substances were induced in this particular industrial plant and therefore bacteria which were also able to degrade testosterone adapted. Lee & Liu (2002) go a step further and conclude that such an estrogen degrading bacteria population probably also adapted near WWTP effluents. Depending on the property of the WWTP concentrations in the effluent are in the ng/L range (see table 1). These amounts could then be degraded near the effluent by these adapted populations.

3. Metabolic pathways and cometabolism

3.1. Metabolic pathways of microbial testosterone and estrogen degradation

As mentioned in section 1.1 the impact of testosterone on the environment is not yet clear as few studies were done investigating the fate of the male sexual hormone in the environment (e.g. Casey et al. (2004), Layton et al. (2000)). Nevertheless, the knowledge of a degradation pathway has been existing for quite a long time. Surprisingly nearly the same amount of testosterone pathway studies in comparison with estrogen pathway studies can be found.

Degradation pathway of testosterone

The study of Kieslich (1985) was the first that stated a whole degradation pathway of testosterone (figure 3). Unfortunately it is not clear from where data were taken. However, Sih for example did a lot of studies in this field (e.g. Sih et al. (1965), Sih (1962)). This so called *9,10-seco pathway* (Fahrbach 2006) was also found in the bacterium *Comamonas testosteroni TA441* (Horinouchi et al. 2003). Initial to the pathway shown in figure 3 testosterone is oxidised to 4-androstene-3,17-dione (analogue to the oxidation of E2 to E1). The most important step of this pathway is the hydroxylation of the C-9 (step **1**) followed by the breakup of the C-C bond between C-9 and C-10, thus the cleavage of ring B (step **2**) and the formation of 3-hydroxy-*9,10-seco*androsta-1,3,5(10)-triene-9,17-dione. This step gave the name of the *9,10-seco pathway*. After that, the cleavage of ring A occurs. The steps **10** to **13** are still controversial (Fahrbach 2006). Except for this, the pathway is well defined.

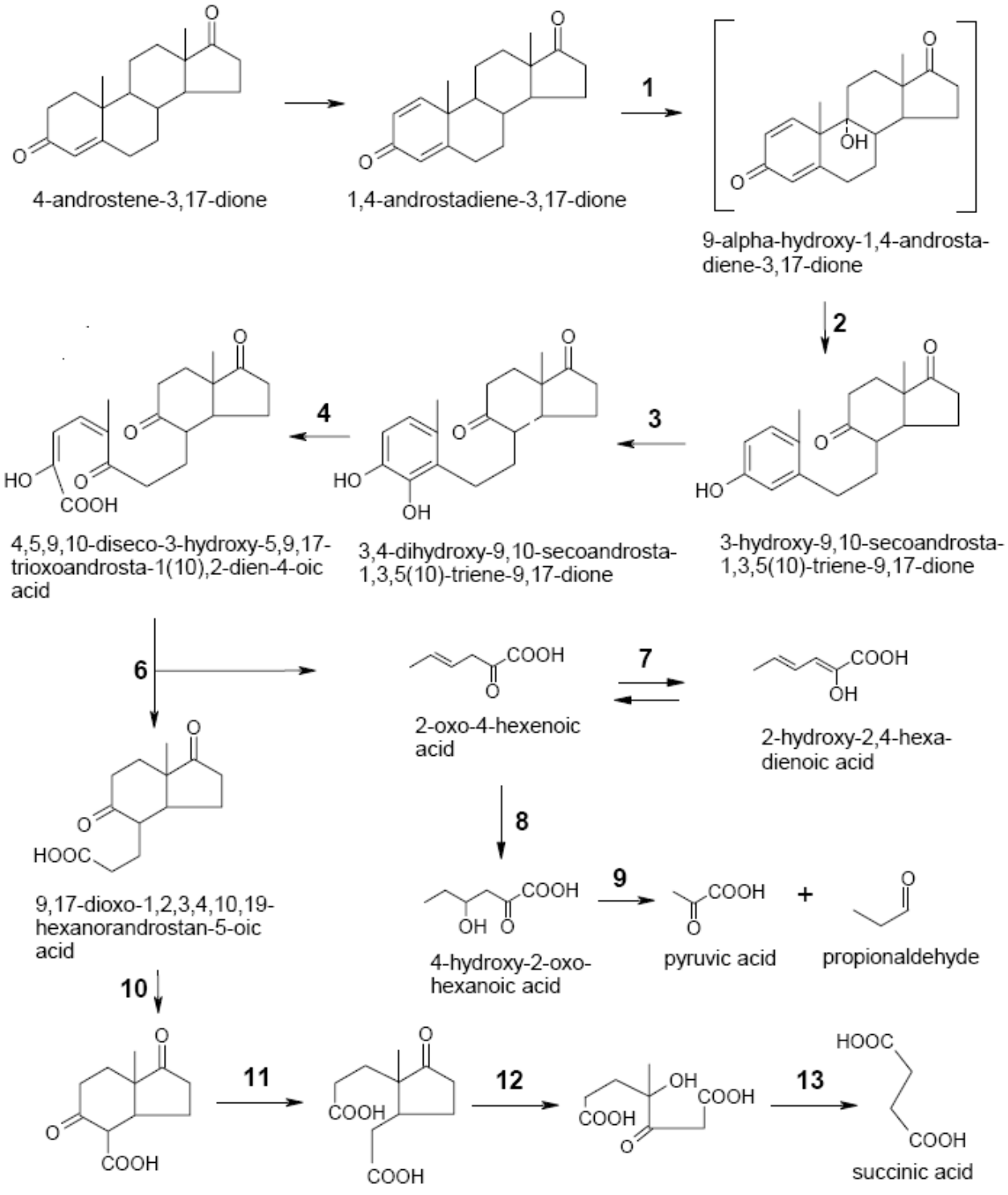

Figure 3: Proposed degradation pathway of testosterone. The first step of the testosterone degradation to 4-androstene-3,17-dione is not shown. Figure is taken from (Fahrbach 2006), the pathway was proposed by Kieslich (1985). See description in the text.

Degradation pathways of E2 and E1

On the other hand, the degradation pathways of estrogens are still not yet thoroughly described. While a lot of studies were made to assess the degradation rate of estrogens, hardly any experiments deal with the question of the exact pathway or intermediate products. It is hence not yet clear how E2 is degraded. As mentioned in section 1.2, oxidation of E2 to E1 is accepted to be the first step of the degradation pathway. More than 40 years ago, Coombe et al. (1966) proposed a pathway by incubating *Nocardia* (isolated from soil) in a mineral salt medium with E1 as a sole carbon source. This pathway is shown in figure 4. The detection was mainly made by UV, MS and NMR. The most important step is the cleavage of ring A to form the product **I** which is catalised by a dioxygenase. The product **I** was not detected. However, this step was proposed because the pyridine carboxylic acid (**II**) which was detected can only be synthetisied via the intermediate **I**. Another reason was that further degradation to **IV** via **III** (these intermediates were also detected) is also consistent with the formation of **I**. However, degradation from **I** to **IV** could not be determined exactly. It was supposed that ring B is the second to be cleaved. In addition, further degradation from **IV** to CO_2 was not shown. The fact that ring A is cleaved at first is supported by the results of Jurgens et al. (2002) and Layton et al. (2000). Jurgens et al. (2002) took river samples and radiolabeled the C-4 atom of spiked E2 with ^{14}C to investigate the $^{14}CO_2$ release after a certain incubation period. It was found out that $^{14}CO_2$ was formed but in a much slower rate than the oxidation from E2 to E1. In figure 4 it can be seen that in step 5, a CO_2 at position 4 is released which is consistent with the findings of Jurgens et al. (2002). Analogue findings were made by Layton et al. (2000) but in the activated sludge of a WWTP.

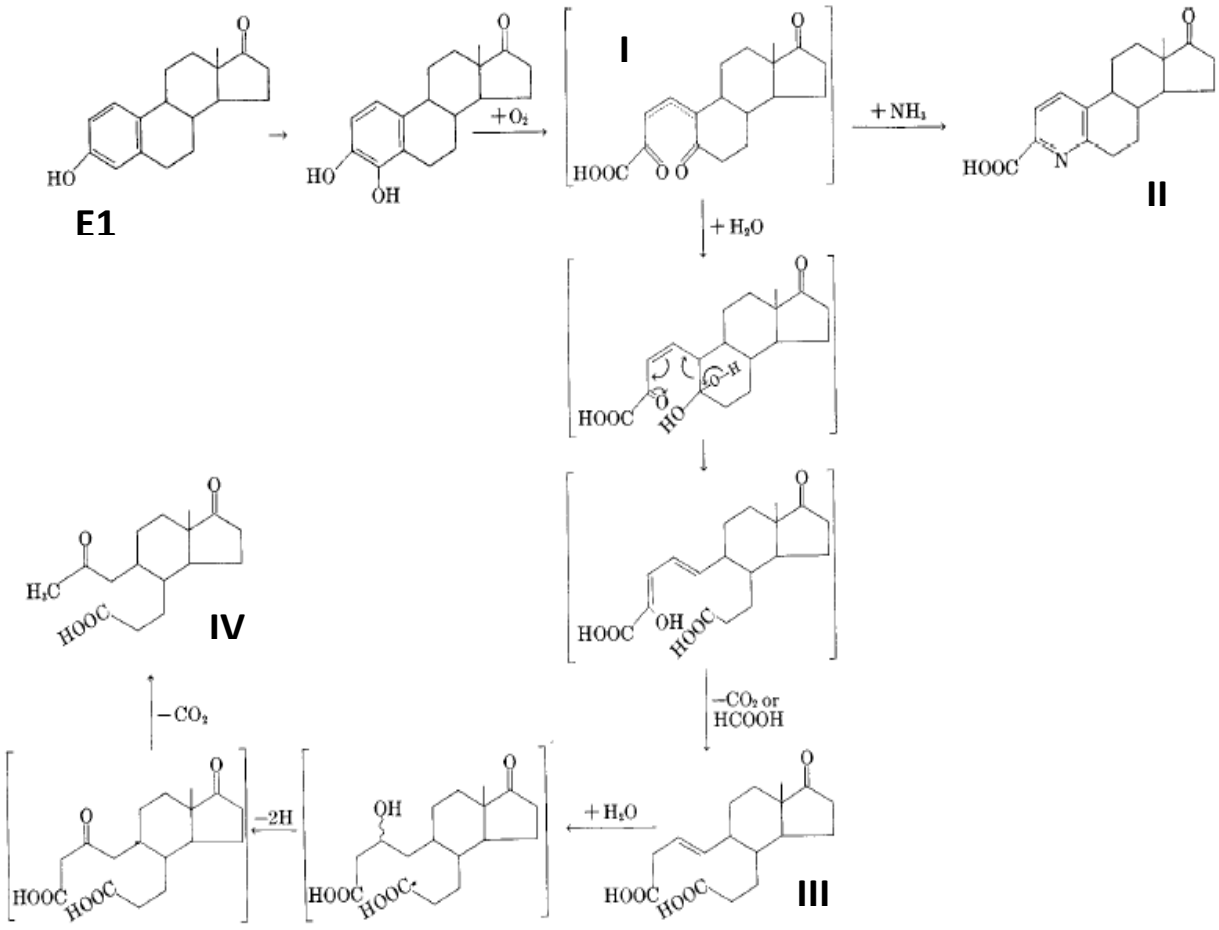

Figure 4: Proposed degradation pathway of estrone (Coombe et al. 1966). See text for explanation.

In contrast, Lee & Liu (2002) proposed a totally different degradation pathway of E2 by investigating supernatant of activated sludge growing on a mineral salt and on high E2 concentrations (figure 5). While E1 was the major product a second intermediate was detected by GC-MS, too. This was determined as a labile lactone (X1). From this intermediate a pathway with the initial cleavage of ring D was proposed. Step 2 of this pathway was supposed because it had been shown several times that hydroxyl acids (figure 5) can be easily transformed to lactones by the release of water. However, it is not said why the further degradation was proposed in that way. Nevertheless, Lee & Liu (2002) states that the role of X1 needs to be further investigated although they claim that in comparison to the results of Coombe et al. (1966) ring A was not the preferred site of attack. Thus, the degradation pathway of E2 is not yet clear. Both mechanisms may work, perhaps depending on the environmental conditions and depending on the bacteria strain.

Degradation pathways of EE2

A very peculiar degradation pathway for EE2 was proposed by Ren et al. (2007) (pathway not shown). Firstly it was suggested that EE2 was degraded to E1 in a first step. However, this is very unlikely because the ethinyl group at C-17 makes this position very recalcitrant to microbial attack. At second, an analogue pathway to the one of testosterone with the initial cleavage of ring B (see figure 3) was shown for the further degradation of E1. This cleavage is however not possible if the C-10 is not methylated as it is in the case of testosterone (Fahrbach 2006). In addition, Coombe et al. (1966) already quoted a paper from Sih and Wang (1963) which found out that organisms doing the *seco-pathway* were not able to metabolise E1. This means that bacteria which are degrading EE2 to E1 in a first step (if they really existed) should not be able to degrade E1 in this way. It could rather be that these fragments were formed in the MS and were misleadingly interpreted as intermediates.

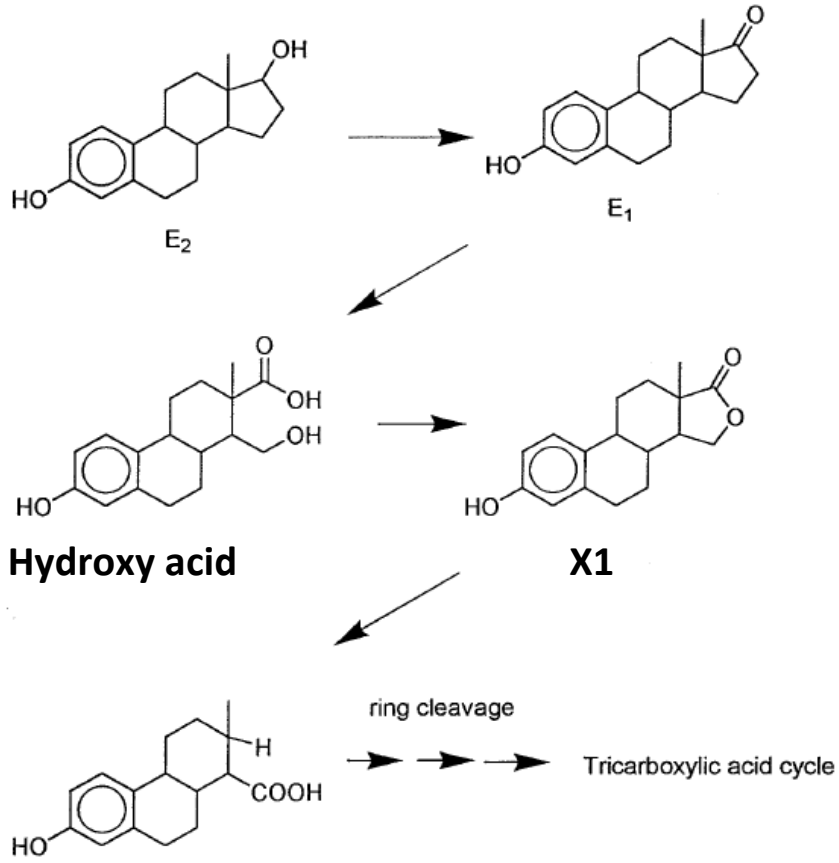

Figure 5: Proposed degradation pathway of estrone (Lee & Liu 2002). See text for explanations.

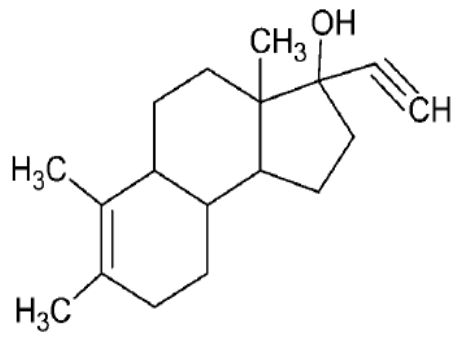

Figure 6: Structure of ETDC, a proposed intermediate of EE2 (Ye & Harper 2007)

Yi & Harper (2007) were interested in the pathway of the cometabolic transformation of EE2 of an enriched nitrifying community from a WWTP (see also section 3.2). They suggested that the molecule is probably attacked at the aromatic ring A where the electron density is highest. There, the π-electrons are susceptible to an electrophilic substitution. It was found by NMR that the formation of a dihydroxy-EE2 (cf. section 2.2 and Arcand-Hoy et al. (1998)) or the cleavage of ring A could be the first step of the EE2 degradation. In contrast to the ring A cleavage of E1 (Coombe et al. 1966), ring A of EE2 was assumed to be cleaved between C-1 and C-2 as well as between C-3 and C-4 to form ETDC (see figure 6). However, these findings are not certain as former studies showed that the ammonium monooxygenase (i.e. the enzyme which is expected to be responsible for this cleavage, see section 3.2) is inhibited by acetylene which also contains a C-C triple bond like EE2. On the other hand, this could also be evidence that the cleavage starts at the ring A because this is the opposite site of ring D (which contains the ethinyl group) which is transformed. In addition, the relatively small acetylene cannot really be compared with a large steroid hormone and it was not clear at which concentration acetylene inhibited the enzyme. Vader et al. (2000) claimed that the degradation of EE2 leaded to hydrophilic substances which can be assumed to be hydroxylated derivatives. It was shown that such substances have a much lower estrogenic activity than the starting product. In the discussion of the degradation pathway of E2 it was never argued that ring A could be most vulnerable due to its highest electron density. This could be a new approach for a unknown degradation pathway or a further argument that the ring A cleavage supposed by Coombe et al. (1966) could be the most likely pathway.

To sum up, the degradation pathway of testosterone is well understood and is called the *9,10-seco pathway.* On the other hand, degradation pathways of the natural estrogens as well as of EE2 are still controversial. For both compounds, an initial cleavage of the ring A (by different ways) is mostly supported either by radiolabelled substances or by an electron density approach. All these degradation pathways are only valid under aerobic conditions. The breakup of C-C bonds and the oxidation of quaternary carbon atoms are dependent on oxygen (Fahrbach 2006). It is not yet clear how the degradation pathway looks like if oxygen is absent though it was shown that steroids can be degraded under such conditions (see section 2.3). Besides energy gaining anaerobic degradation of steroids (e.g. found by Fahrbach (2006)) cometabolic pathways could also play a role in the degradation of these substances. Such cometabolic pathways are the topic of the next section.

3.2. Cometabolic steroid degradation by nitrifying microorganisms

The degradation pathways shown in section 3.1 were mainly observed in experiments of an isolated bacterium living on high steroid concentrations. However, in the environment and in the WWTP much lower steroid hormone concentrations are available. It is not sure whether the steroids at these low concentrations are degraded with the same pathways. One way to be considered is the cometabolic pathway. This means that the estrogens are incidentally introduced in a rather unspecific enzyme where they are transformed into a metabolite. The

bacteria do not gain any energy by this process. Andersen et al. (2003) for example found that EE2 degradation in the nitrification tank plays a major role in the removal of these endocrine active substances. Many studies showed that the ammonium monooxygenase (AMO) (which is an enzyme that only nitrifying bacteria (e.g. *Nitrosomonas europaea*) contain) is able to cometabolise different organic substances.

Vader et al. (2000) firstly suggested that AMO can be the key enzyme of the EE2 degradation in the nitrification tank. It was found that an enriched nitrifying sludge (which was living on ammonium as the sole energy source) could degrade EE2 in presence of a high NH_4^+ concentration. Half-life was measured to be around 24 hours. However, sludge with a small amount of ammonium oxidising bacteria could not degrade any EE2. Hence, it seemed that ammonium oxidising bacteria were responsible for the EE2 removal. In addition to this, degradation products were found to be more polar than the steroid itself. This is quite reasonable as it is known that monooxygenases introduces oxygen into C-H bonds. Vader et al. (2000) therefore concluded that nitrifying bacteria are a sink for EE2 in the natural environment as well as in the WWTP. Based on these findings, Shi et al. (2004) made experiments with nitrifying activated sludge (NAS) and with the ammonium oxidising bacterium *Nitrosomonas europaea*. They showed that E2 could be removed very fast (half-life of 0.5 hours) by NAS while the other estrogens (E1 and EE2) had longer half-lives (12-23 hours). The experiments were carried out with much higher estrogen concentrations than they occur in nature or in the WWTP. E2 was suggested to be oxidised to E1 and this was further degraded to unknown but more polar substances (cf Vader et al. (2000)). All estrogens could also be removed by the pure *N. europaea* culture at similar rates. However, *N. europaea* did not degrade E2 via E1. Because the degradation step was different either NAS or *N. europaea* was studied it was concluded that the oxidation of E2 to E1 and the further degradation of E1 in the NAS was done by other (probably heterotrophic) bacteria. In addition to this, Shi et al. (2004) inhibited the AMO in the NAS as well as in the pure *N. europaea* culture. They found that EE2 was still reduced in the NAS while *N. europaea* could not degrade EE2 anymore. That deduced again that heterotrophic bacteria could degrade such estrogens, too. Nevertheless, they found that the ammonium monooxygenase is able to cometabolise estrogens and plays an important role in the degradation of estrogens in certain ammonium oxidising bacteria. Yi & Harper (2007) found linear relationship between EE2 and NH_3 removal. Therefore it was concluded that ammonium oxidising bacteria are able to degrade steroid hormones. However, the authors state that the heterotrophic bacteria need to be considered as well since they also possess several different mono- and dioxygenases. As this linear relationship is only a correlation but no causality can be drawn, it is also possible that enzymes of heterotrophic bacteria could have leaded to the formation of the analysed transformation products (Yi & Harper (2007) worked with an enriched nitrifying microbial community and not with isolated cultures).

Nevertheless, a recent study criticised a lot of these findings (Gaulke 2008). The main point was that for all experiments much higher estrogen and ammonium concentrations were used than they occur naturally or in a normal WWTP influent. Gaulke (2008) therefore did experiments with reasonable input concentrations. Pure *Nitrosomonas europaea* and *Nitrosospira multiformis* cultures were tested to grow on environmental relevant concentrations and it was found that no EE2 removal at all occurred (although ammonium was oxidised to nitrite). Therefore experiments were done with higher ammonium but still low EE2 concentrations and a transformation of EE2 was observed. However, the only metabolites turned out to be abiotical formed nitro-EE2. Due to the large production of nitrite by the ammonium oxidising bacteria nitrite could react with the EE2. Thus, it was concluded that enzymatic processes were not responsible for any transformation of EE2 neither in this experiment nor in previous experiments. Gaulke (2008) stated that nitrification is not an

important process for the removal of estrogens. Heterotrophic bacteria are rather considered to be the main sink for these substances. On the other hand, a recent study (Forrez et al. 2009) which considered the degradation of EE2 in the nitrification tank concluded again that ammonium-oxidising bacteria play a very important role.
To sum up, it is not yet clear which kinds of bacteria are important for the cometabolic degradation of estrogens. It was shown that estrogens can be reduced in pure nitrifying bacteria strains. However, it is still controversial if the removal was abiotical or induced by the AMO. On the other hand heterotrophic bacteria must be able to transform these hormones, too. This is due to the fact that these substances were still reduced in enriched nitrifying cultures when AMO was inhibited. For further research it is important to find out which microbes play the biggest role for the degradation in the WWTP.

4. Conclusion and outlook

Several investigations showed that naturally occurring steroid hormone concentrations can occur in the ng/L range. Such hormones cause endocrine disruptions in fish at environmental concentrations (see section 1.1). The input of steroid hormones (mainly via the WWTP) should therefore be reduced. Although E2 is fast degraded to E1, this does not reduce the estrogenic potential a lot. Since E1 is quite persistent in the environment, major effects are expected to be from E1. In addition, EE2 is even more recalcitrant because it contains an ethinyl group at the same C-atom which possesses a hydroxyl group that is normally vulnerable to microbial attack. Hence, EE2 is also thought to accumulate in the environment and can cause effects in the same range like E1 although it is secreted in a much lower extent. The risk of effects is highest where WWTP effluents are rarely diluted (Galli & Braun 2008) and in anaerobic sediments where the degradation rate is much smaller than under aerobic conditions (see section 2.4). Most important degradation process is the degradation by microorganisms. Degradation rates in general are higher at higher temperatures following the rule of Arrhenius (c.f. section 2.2). This means for example that degradation in rivers is much better in summer than in winter (c.f. section 2.1). Concerning this, there is a problem in the WWTP. Although temperature differences between summer and winter are smaller than in natural waters, slight decreases of the temperature can have a big effect on activated sludge forming bacteria. This is most problematic for slow growing bacteria like nitrifiers which are often washed out during winter months. If these bacteria really play a significant role in the degradation of steroid hormones (which in not yet clear, see section 3.2), an increase in the sludge retention time (SRT) would be most efficient. Layton et al. (2000) for example showed that WWTP that failed to nitrify also failed to remove EE2. It was argued several times that an optimisation of the SRT as well as of the hydraulic retention time (HRT) would be the best solution in order to properly remove estrogens in the WWTP (De Mes et al. (2005), Koh et al. (2008) and Vader et al. (2000)). In contrast, Leusch et al. (2005) found that an increase of the SRT hardly had an effect on the removal rate of steroids and it was claimed that a tertiary treatment step (c.f. section 1.2) could be more efficient. Mainly ozone oxidation resulted in a satisfying removal of steroids (De Mes et al. 2005). Nevertheless, De Mes et al. (2005) and Koh et al. (2008) argued that these steps are very cost intensive as well as energy consuming. Thus, they are not only economically unfeasible but they also damage the environment due to the high energy use. Membrane bio reactors (MBR) are also considered to be more efficient than the activated sludge method (Joss et al. 2004). However, it is not possible to alter all consisting plants in a MBR and in addition to this, the MBR has other disadvantages compared with the activated sludge method. Another problem is that estrogens enter the WWTP in conjugated forms to a large extent. As it can take long

time to deconjugate these substances, there is not enough time in the treatment step to subsequently degrade the free estrogens. In further studies, an eye should be put on the fate of conjugated estrogens within the WWTP.
Although a lot of research has been done to determine removal rates of estrogens, their degradation pathway is still controversial. The first step, i.e. the oxidation from E2 to E1, is widely accepted. Nevertheless, it is still unknown at which ring the cleavage initially occurs. Further studies are required to determine this pathway. However, this is not an easy task as it is difficult to identify metabolites. For testosterone it can be argued that the opposite is true. While the degradation pathway via the *9,10-seco pathway* is widely accepted there is not a lot of research concerning testosterone concentrations and degradation rates in the WWTP and in the environment. More effort should be put in the investigation of testosterone as endocrine disrupting effects can also come from the male sexual hormone. In addition to this, it is not yet clear which step of the waste water treatment is most important for the removal of any steroid hormones. Nitrifying bacteria are thought to be much efficient in removing EE2 cometabolically by the AMO. However, it was recently shown that the observed degradation products were nothing but abiotical formed nitrated conjugates of EE2 which were misleadingly interpreted as cometabolically produced intermediates in the previous experiments. Further studies are required to verify the formation of the nitro-EE2 or to prove that ammonium-oxidising bacteria are actually very important in the EE2 removal. Several studies claim that heterotrophic bacteria in the aerated tank are most efficient in the estrogen removal and other experiments showed that the denitrification tank can act as a sink for steroid hormones. Bacteria that can degrade steroid hormones metabolically under denitrifying conditions could be isolated. It is however not certain if these bacteria have a big influence in removing estrogens or if other bacteria in the denitrifying tank are more efficient.

The knowledge of the most efficient part of the WWTP would help improving the treatment of waste water, mainly with respect to SRT, HRT and microbial community. Nevertheless, it is difficult to find out which organisms are most efficient in removing steroids. It is even difficult to investigate which organisms are most abundant in the activated sludge because sludge composition varies a lot. It is of highest importance to improve WWTP with the simplest method possible in order that it is feasible not only to adapt large plants in industrialised countries but also to adapt small plants as well as plants in developing countries. The improved removal of steroid hormones in WWTP is the key step in reducing endocrine disruptions of aquatic organisms.

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