Fate of estrogens in biological treatment of concentrated black water

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Dit onderzoek is uitgevoerd binnen de onderzoekschool SENSE (Socio-Econmic and Natural Sciences of the Environment).

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op vrijdag 2 november 2007
des ochtends te elf uur in de Aula

Author: De Mes, T.Z.D.
Title: Fate of estrogens in biological treatment of concentrated black water
ISBN: 978-90-8504-753-7
Publication year: 2007
Subject headings: Estrogens/ Black Water/ Wastewater /Biological treatment

PhD-Thesis Wageningen University, Wageningen, The Netherlands - with references - with summaries in English and Dutch- 154 p.

Abstract

De Mes, T.Z.D. Fate of estrogens in biological treatment of concentrated black water. PhD thesis, Wageningen, the Netherlands.

Sewage treatment plants (STPs) effluents were found to have estrogenic character which is mainly due to the presence of estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2). E1 and E2 are natural hormones excreted by mammals in urine and faeces, whereas EE2 is a synthetic hormone present in the contraceptive pill. The largest part of the estrogens is excreted via urine as glucuronide or sulphate conjugate with no estrogenic properties. These conjugates can be cleaved back to their original form by bacterial enzymes, where deconjugation of glucuronide conjugates is faster than of sulphate conjugates.

Human excreted estrogens are insufficiently removed in conventional treatment systems designed to remove bulk organic matter and nutrients. Maximum reported concentrations in STP effluents are 76 ng/l for E1, 64 ng/l for E2 and 42 ng/l for EE2. Effects on fish were already prevalent at 3.3 ng E1/l, 1 ng E2/l and 0.03 ng EE2/l, values that are often exceeded in surface waters.

Present research focused on the fate of estrogens in sanitation concepts with source separated collection and treatment of domestic wastewater i.e. black water (toilet), grey water (personal hygiene, kitchen) and rain water. Source separated collection offer benefits in terms of energy and resource conservation. By applying vacuum toilets in these concepts, the black water volume containing estrogens is about 7 L per person per day, whereas in conventional sanitation concepts this volume (including rain water) is about 200 L per person per day.

Adsorption and biodegradation are considered the most important processes for the removal of estrogens in biological wastewater treatment systems, and therefore adsorption constants and first order degradation rates were determined. Besides, their fate was investigated in a concentrated black water pilot treatment system consisting of UASB septic tank, with aerobic post-treatment. Determination was established by HPLC with UV, fluorescence and diode array detection and GC-MS for environmental relevant concentrations.

The highest degradation rates were obtained under aerobic redox conditions for all three estrogens, with EE2 being the most persistent as was also confirmed in literature. Increasing sludge retention time had a positive effect on the biological removal rates, whilst nitrifiers did not contribute significantly to it. The results showed that reduced bioavailability, e.g. desorption of adsorbed estrogens, can suppress the conversion rate. During anaerobic conditions, a reduction of E1 to E2 was observed but no decline for the sum of E1 and E2 nor EE2 was observed in various types of sludge.

In the pilot UASB septic tank effluent, total concentrations of 4.02 μg E1/l and 18.69 μg E2/l, of which >70% for E1 and >80% for E2 were in conjugated form. EE2 concentrations were below the detection limit. In the effluent of the post-treatment E1 and E2 were present in concentrations of 1.37±1.45 μg/l and 0.65±0.78 μg/l, respectively. The limited deconjugation of conjugated estrogens during treatment was demonstrated when UASB septic tank was spiked with the sulphate conjugate of E2, revealing that 99% of the detected E2 in the final effluent is in conjugated form.

Even though the application of source separation can prevent storm water overflow and reduce the volume of the wastewater in which estrogens are present, high effluent concentrations after biological treatment necessitate additional treatment. Besides, there is a need for information on the deconjugation rates and the behaviour of conjugated estrogens in general.

Keywords: estrogens, black water, wastewater, biological treatment.

Voor mijn familie.

Contents

Abbreviations

Ace	Acetone
AOP	Advanced oxidation process
AS	Activated sludge
ATU	*N*-Allylthiourea
BOD	Biological oxygen demand (mg/L)
CENSA	Central sanitation concept
CFU	Colony forming units
COD	Chemical oxygen demand (mg/L)
DAD	Diode array detector
DeSaR	Decentralised sanitation and re-use
DHS	Downflow hanging sponge reactor
E1	Estrone
E2	17β-Estradiol
E2-α	17 α-Estradiol
ECD	Electron capture detection
ED	Endocrine Disrupting
EE2	17α-ethynylestradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FLU	Fluorescence
GAC	Granular activated carbon
GCMS	Gas chromatography mass spectrometry
GCMS-EI	Gas chromatography with mass spectrometry an electron impact
GCMS-MS	Gas chromatography-tandem mass spectrometry
GCMS-NICI	Gas chromatography negative chemical ionisation mass spectrometry
GC-NCI-MS	Gas chromatography negative-ion chemical ionisation mass spectrometry
GS	Granular sludge
HGRGC-MS-MS-SIM	High resolution gas chromatography with mass spectrometry operating in single-ion monitoring
HPLC	High pressure liquid chromatography
HRGC-(NCI)-MS	High-resolution gas chromatography with negative chemical ionisation mass spectrometric detection
HRT	Hydraulic retention time
IS	Internal standard
LC-APCI-MS-MS	Liquid chromatography atmospheric pressure chemical ionisation tandem mass spectrometry
LC-DAD	Liquid chromatography with diode array detection
LC-DAD-MS	Liquid chromatography with diode array detector and mass spectrometry
LC-ESI-MS-MS	Liquid chromatography electorspray ionisation tandem mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
LC-MS-MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limits of quantification
M	Molair (mole/l)
MBR	Membrane bioreactor
MBR-S	Membrane bioreactor sludge
$MeCl_2$	Dichloromethane

MeOH	Methanol
MF	Micro-filtration
MIEX	Magnetic ion exchange resins
MTBSTFA	*N*-methyl-*N*-tertbutyldimethylsilyltrifluoracetamide
NF	Nano filtration
NOEC	No observed effect concentration
OECD	Organisation for economic co-operation and development
PAC	Powdered activated carbon
PM	Pig manure
RIA	Radioimmunoassay
RO	Reversed osmosis
SBR	Sequencing batch reactor
SBR-S	Sequencing batch reactor sludge
SDB	styrendivinlybenzene
SIM	Selected ion mode
SPE	Solid phase extraction
SRT	Sludge retention time
SS	Suspended solids
STP	Sewage treatment plant
TBDMCS	Tertbutyldimethylchlorosilane
TOC	Total organic carbon
TS	Total solids
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket
UASB-S	Upflow anaerobic sludge blanket sludge
UF	Ultra-filtration
UV-VIS	UV-visible
VS	Volatile solids
VSS	Volatile suspended solids
YES	Yeast estrogen screen

Nomenclature

C_s	Concentration in the solid phase (mg/kg)
C_w	Concentration in the water (liquid) phase (mg/l)
$C_{w,0}$	Concentration in absence of organic colloids (mg/l)
H_c	Henry's law constant (atm.m3/mole)
K_d	Sorption coefficient (l/kg)
K_f	Freundlich adsorption coefficient (l/kg)
K_{oc}	Organic partition coefficient
K_{ow}	Octanol water partition coefficient
k_{sor}	Adsorption rate (d-1)
k	First order degradation constant (d-1)
n_f	Freundlich sorption constant (-)
S	Water solubility (mg/L)

Chapter 1

General introduction

Abstract

This thesis focuses on the fate of three estrogens, comprising the natural estrone (E1) and 17β-estradiol (E2) and the synthetic 17α-ethynylestradiol in wastewater treatment systems suitable within the Decentralised Sanitation and Reuse (DeSaR) concept. In many aspects DeSaR-concepts offer advantages over current wastewater treatment, but the reuse aspect necessitates research on the removal of hazardous compounds. The selected compounds are pointed at to be responsible for a large part the estrogenic character of sewage treatment plant (STP) effluents. As STPs are unable to fully eliminate these compounds, a solution involves minimising the problem by source separation. Black water (toilet), containing virtually all estrogens, is separately collected from other wastewater streams produced in the household. The black water volume is small, and the concentration in terms of chemical oxygen demand (COD), nutrients and estrogens is high, so treatment can be optimised to remove micro-pollutants.

1.1 Scope of the thesis

The decentralised sanitation and reuse (DeSaR)-concept focuses on the separate collection and treatment of different domestic wastewater-streams, and is an innovative concept and substitute for the present central sanitation concept (CENSA). This PhD-research was part of a multidisciplinary research on DeSaR, which was a continuation after a positive outcome on research on DeSaR for large buildings (EET-kiemproject, 2000). The overall objective of the multidisciplinary research was to obtain sufficient knowledge to facilitate application of such a concept in an environmentally and hygienically sensible manner.

As the presence of estrogenic compounds is a recognized environmental problem due to incomplete removal in CENSA, there was a necessity to study the fate of such compounds in DeSaR. Therefore, three of the most relevant compounds were selected for research.
This developed into the overall scope of this thesis; to investigate the fate of estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) in DeSaR-concepts.

1.2 Estrogens as an environmental problem

Currently, large numbers of substances have been identified affecting the endocrine system of humans and animals, and the list continuously grows. Endocrine disruption as an environmental health issue was first noted by the US Environmental Protection Agency in 1996 (Barlow, 2001), after first alarming messages on hermaphrodite fish caused by compounds present in sewage treatment plant (STP) effluents came from the UK and dated back to 1993 (Ends, 1993). However, the first signs of concern dated from 1962 in the publication "Our stolen future" by Theo Colborn. There are two types of endocrine disrupters (EDs): The first comprises of natural hormones, including the estrogens, progesterone and testosterone, but also phyto-estrogens, which are present in some plants. The second include man-made substances, able to mimic the estrogenic effects, referred to as xeno-estrogens, which include synthetic hormones, and a large range of chemicals present in industrial detergents, pesticides and plastics. A comprehensive study was conducted in the Netherlands to determine the prevalence of (xeno-) estrogens in the aquatic environment. The study was

initiated in 1997 by the directorate-general for public works and water management and was performed during 1999-2001 (Vethaak *et al.*, 2002).

Sewage treatment plants (STPs) were found to be an important point source for EDs in the environment. Many of the xeno-estrogens are present in wastewater treatment effluents in the μg/l range, whereas natural estrogens were only present in the ng/l range. Although some of the xeno-estrogens appeared on the priority list of the water framework directive, like brominated diphenylethers, nonlyphenols, octylphenols, and tributyltin (EU, 2001), xeno-estrogens only contribute approximately 1- 4% to the total estrogenic activity of domestic wastewater (Körner *et al.*, 2000). Three sterols, the natural hormones 17β-estradiol (E2) and estrone (E1) and the synthetic hormone 17α-ethynylestradiol (EE2), used in the contraceptive pill, were isolated from effluents of domestic STPs and identified as prime contributors to its estrogenic character (Desbrow *et al.*, 1998; Körner *et al.*, 2001; Onda *et al.*, 2003; Routledge *et al.*, 1998). Although these three compounds are not included in the final list of priority substances identified by the EU water framework directive (EU, 2001), these compounds need attention, as confirmed by many researchers who recognized a potential risk to the aquatic environment (Knacker, 2002; Bursch *et al.*, 2004; Ternes *et al.*, 2004).

1.3 Directions towards a solution

The presence of estrogens in surface waters is mainly attributed to the insufficient removal during sewage treatment. As they are detected in very low concentrations they are designated as micro-pollutants, which is also the case with pharmaceuticals. In fact pharmaceuticals and estrogens are often studied at the same time. According to Larsen *et al.* (2004), four methods have been identified to remove these micro-pollutants from wastewater:

1. optimizing existing treatment technology,
2. upgrading existing treatment plants with new end of the pipe technology,
3. source control measures,
4. source separation methods.

In general the first two options are embraced by governmental institutions in order to comply with the water framework directive, as they do not require extensive changes or capital investment in the current infrastructure. An example of source control measures involves the introduction of an environmental classification system for pharmaceuticals as done in Sweden, in cooperation with industry (MPA, 2004). Labelling allows physicians and patients to choose the pharmaceutical with the least negative impact on the environment. Also measures can be taken during the industrial production of pharmaceuticals to reduce unnecessary product losses. However, main part of the estrogens are naturally excreted by humans. Therefore, source separation measures can be seen as a sustainable solution for the entire wastewater problem (Larsen *et al.*, 2004). It is estimated that 70% of pharmaceuticals are likely to be present in urine, and 30% in the faeces (Alder *et al.*, 2006). The most drastic source separation measure is to separate the collection of urine, in which the concentration of estrogens will be 100-500 times higher than found in sewage. Even when both urine and faeces are collected using water saving vacuum toilets, estrogens can be expected at 30 times

higher concentrations as compared to present situation. Separate collection of urine plus faeces enables the collection of nearly all natural and synthetic estrogens within a very small volume.

2.4 Decentralised Sanitation and Reuse (DeSaR)

DeSaR-concepts are applicable at any scale (Zeeman and Lettinga, 2001) and their advantages over CENSA are numerous. Community on-site sanitation concepts minimize the need for large sewer systems. Wastewater-streams are separated according to their origin, composition and degree of contamination, and for each stream an appropriate treatment can be applied. Often used synonyms for DeSaR, describing the same concept, are source separated sanitation and ecosan. Figure 1.1 illustrates the DeSaR concept and the different (waste)water stream, which can be distinguished in black water (urine and faeces), grey water (kitchen, laundry and personal hygiene) and rainwater. The stream causing most serious hygiene problems, urine plus faeces, can be significantly reduced in volume by using water saving toilets (Figure 1.1). The high concentration in terms of chemical oxygen demand (COD), as a result of the reduced volume, enables the application of anaerobic digestion systems. Anaerobic wastewater treatment is seen as the core technology in DeSaR for the treatment of concentrated black water. Optionally, organic kitchen waste can be co-digested in the anaerobic system. Overall, DeSaR aims at a sustainable collection, transport and treatment of domestic waste (water) streams, focussing on the prevention of clean water consumption, recovery of resources and energy and reduction of emissions of hazardous compounds. Research conducted so far on the occurrence and behaviour of estrogens dealt mainly with "conventional" collection and treatment methods, commonly where urine and faeces become diluted into a volume of 200 liter per person per day.

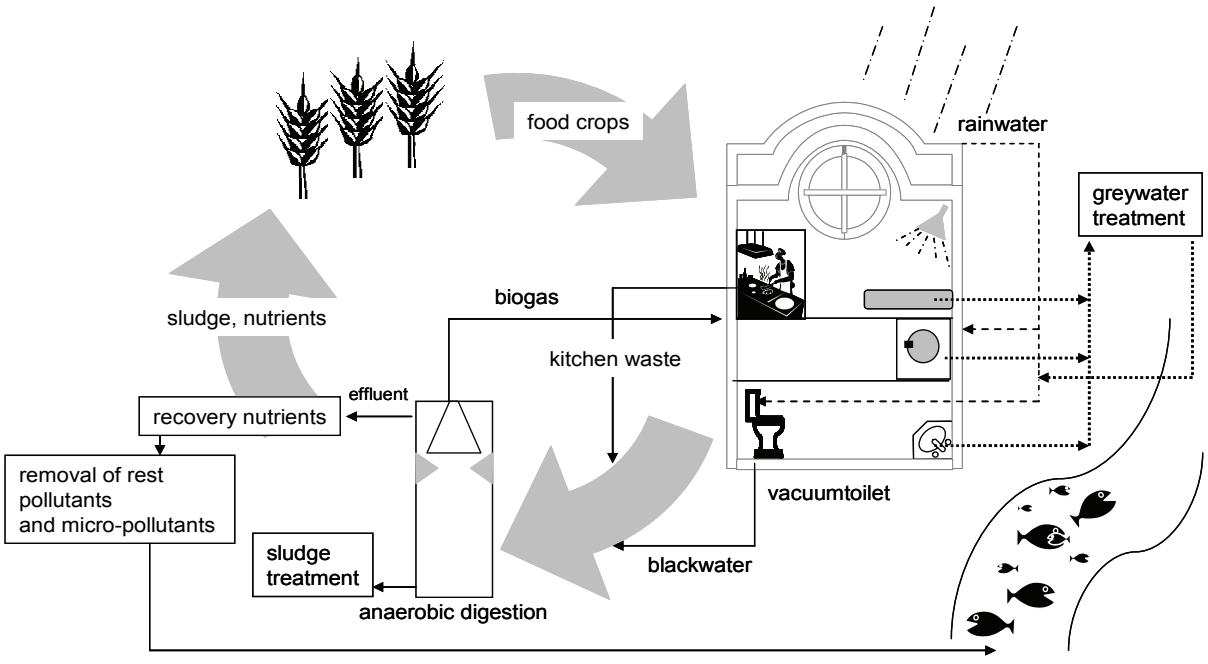


Figure 1.1. Schematic view of the DeSaR-concept.

1.5 Outline of the thesis

The research is following an hourglass pattern, as can be seen in Figure 1.2. At first an inventory of current knowledge was produced concerning the fate of E1, E2 and EE2 in existing wastewater treatment systems, in the form of a literature research (Chapter 2).
The large challenge on developing suitable analytical techniques for the determination of E1, E2 and EE2 in the complex black water matrix, is extensively described in Chapter 3. Then the specific fate of E1 and E2 under different redox conditions as occurring within DeSaR systems, using lab-scale batch experiments, is described in Chapter 4. Chapter 5 focuses on the degradation of EE2 under different redox conditions. In the middle of the hourglass, the behaviour of E1, E2 and EE2 in a pilot-scale black water treatment is researched and presented in Chapter 6. Chapter 7 comprises a general discussion of the results of the research and the consequences for the application of DeSaR-systems.

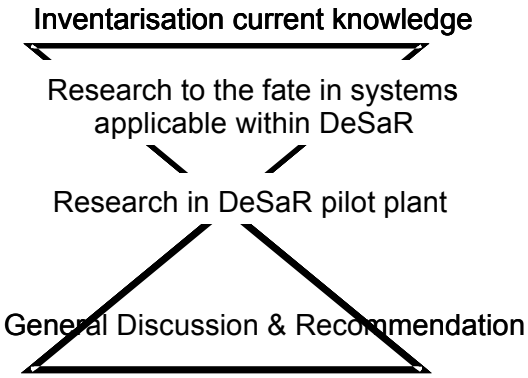


Figure 1.2. Set-up of the thesis.

Chapter 2

literature review: Occurrence and fate of estrone, 17β-estradiol and 17α-ethynylestradiol in STPs for domestic wastewater

Redrafted after publication in Reviews in Environmental Science and Bio/Technology (De Mes *et al.,* 2005)

Abstract

Estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) discharged from sewage treatment plants (STPs) into surface waters, are seen as a threat affecting aquatic life by their estrogenic character. Therefore considerable research was conducted on the fate and removal of these compounds. Since these compounds are present in influents and effluents in the ng/l range, methods for detection deserve special attention. Most important processes that play a role in the removal of estrogens are: adsorption, aerobic degradation, anaerobic degradation, anoxic biodegradation and photolytic degradation. Half-lives tend to vary and are remarkably shorter at low initial concentrations. In general, anaerobic conditions result in long half-lives compared to aerobic conditions. EE2 showed most persistence of the compounds, besides also the estrogenic effect *in vitro* is about 2-3 fold higher compared to E2. The three compounds show higher affinity to sorb to sludge compared to other tested adsorption materials like sediment and soil. Aerobic degradation is by far the most efficient in removing these compounds, but adsorption seems to play a significant role in retaining the estrogens inside full-scale STPs. Removal rates in full-scale plants depend on the hydraulic retention time (HRT), sludge retention time (SRT) and loading rates, but lack of information on the exact dependency so far prevents an optimal design able to fully eliminate estrogens from wastewater.

2.1 Introduction

Due to the proven estrogenic effect of sewage treatment plant (STP) effluents considerable research is being directed towards the occurrence and fate of estrogenic compounds in wastewater. As was mentioned in Chapter 1, three sterols, the natural hormones 17β-estradiol (E2) and estrone (E1) and the synthetic hormone 17α–ethynylestradiol (EE2), were found to be responsible for over 90% of the estrogenic character of domestic wastewater (Körner *et al.,* 2001; Onda *et al.,* 2003). Despite the low contribution of xeno-estrogens in practice (1- 4%) of the total estrogenic activity of wastewater (Körner *et al.,* 2000), a combination of multiple xeno-estrogens can still lead to a dramatic enhancement of estrogenity (Rajapakse *et al.,* 2002). The reason for the estrogenic activity of numerous substances is the relatively low specificity of the human estrogen receptor because the binding pocket is nearly twice as large as the molecular volume of E2 (Brzozowski *et al.,* 1997).

This chapter emphasises on the behaviour of E1, E2 and EE2 in STPs, in order to evaluate current knowledge and to trace where information is lacking. At first the amounts expected to enter STPs as constituents of the wastewater are outlined. Then the necessity of gaining knowledge on this subject is outlined by a description of the environmental consequences and prevalence, followed by a brief description of analytical procedures. Next, different removal processes and the importance of these particular processes for the removal of E1, E2 and EE2 are described. Then the removal efficiencies in current STPs and eventual additional treatment systems are reported. Finally, a conclusion is drawn on current knowledge and suggestions for further research are identified.

2.2 Excretion of estrogens

Estrogens influence growth, development, differentiation and function of peripheral tissues of the female and male reproductive system, such as the mammary gland, uterus, vagina, ovary, testis, epididymis and prostate; and they play an important role in bone maintenance, the

cardiovascular system and the central nervous system (Shimada *et al.,* 2001). Estrogens are produced by human glands, mainly by ovaries and testis, and are excreted within urine and faeces. The synthetic EE2 is the main estrogen used in oral contraceptives, which is the most prescribed drug world-wide (Williams and Stancel, 1996).

Estrogens are metabolised in the liver, where they are enzymatically mediated and conjugated with either sulphate or glucuronide esters to the hydroxyl groups in the C3- and C17-position of the basic steroid structure (Williams and Stancel, 1996). Theoretically there are eight different possible conjugates for E2 and EE2, as they have two hydroxyl groups. E1 has only two possible conjugates (Kjølholt *et al.,* 2004). Conjugation increases the water solubility, which eases excretion, but also makes them more mobile in the environment compared to free hormones. Conjugated hormones with a group on the C3 position are biologically inactive (Ingerslev and Halling-Sørensen, 2003) and do not exhibit estrogenic properties. Estrone is excreted as sulphate- rather than as glucuronide conjugate, as has been found in urine from pregnant women (Andreolini *et al.,* 1987). In human urines, the glucuronides are dominant (male 85%, female 65%), against sulphates (male 15%, female 35%) (Matsuda *et al.,* 2001). Natural hormones are mainly excreted in urine and only a very small fraction in faeces, in unconjugated form. The reason for estrogens being unconjugated in faeces is the ability of bacteria, for instance *E.coli*, to produce the enzyme β-glucuronidase, which can hydrolyse glucuronide conjugates back into their original form (Ternes *et al.,* 1999a; Legler, 2001).

According to Williams and Stancel (1996), the total daily excretion rate of natural estrogens ranges from 10-100 μg for women, 5-10 μg for women after the menopause and 2-25 μg for men. Average excretion values from a study amongst female inhabitants of a Roman condominium were 32 and 14 μg/day of conjugated E1 and E2 respectively (D'Ascenzo *et al.,* 2003). According to Adlercreutz *et al.* (1986) women can excrete about 7 μg of E1 and 2.4 μg of E2 of unconjugated forms daily with urine. Approximately 0.5 μg E1 and 0.4 μg E2 is eliminated in faeces per day (Adlercreutz 1994). Fotsis *et al.* (1980) reported a daily excretion with urine of unconjugated forms of 8.0 μg as E1 and 3.0 μg as E2. Calculations on the percentage contribution to the total excretion of both conjugated and unconjugated natural estrogens and the synthetic EE2, are depicted in Figure 2.1, illustrating that pregnant women contribute the most estrogens to the total excreted amount.

The average daily dose of the synthetic hormone used in the contraceptive pill is 35 μg EE2, taken in during 21 days of a 28 day period (Katzung, 1995). Up to 80% of the administered EE2 is excreted as unmetabolized conjugates (Ranney 1977; Maggs *et al.*, 1983). Of the daily dose, 22 to 50 % of EE2 is excreted via urine of which about 64% is conjugated, and approximately 30% is excreted via faeces (Reed *et al.,* 1972). The oral bioavailability of EE2 is about 42% due to an extensive first-pass metabolism in the intestinal wall and liver (Weber *et al.*, 1996). More than 30% of EE2 is sulphated, which accounts for approximately 60% of the first-pass metabolism (Back et al., 1979; 1982). Only 1-2% of the administered EE2 has been found to be de-ethynylated and transformed to E1, E2 or E3 (Ranney, 1977). In 2001, 43% of the female Dutch population in the age of 16-49 years used

oral contraception (CBS 2002). The contribution of EE2 to the total amount of excreted estrogens is only about 1% (Figure 2.1), but this compound is considerably more persistent in STPs compared to the natural hormones (Ternes *et al.,* 1999a/b). Due to the introduction of the ethynyl-group, the ring becomes extremely stable against oxidation. Thereby EE2 showed the highest estrogenic potency in *in vitro* tests; the potency compared to the other estrogens can be expressed as EE2>E2>E1 (Larsson *et al.,* 1999). It has also been shown *in vivo* tests in fish that , EE2 is 11 to 130 times more potent than E2, while E2 is 2.3 to 3.2 times more potent than E1 (Legler, 2001; Thorpe *et al.,* 2003; Metcalfe *et al.,* 2001). The amount of E2 used for pharmaceutical purposes contributes less than 5% compared with the natural E2 excretion (Christensen,1998).

Rough estimations of the maximum concentration of all natural estrogens present in sewage are about 1 µg/l and about 13.4 ng/l for the synthetic EE2 (Blok and Wösten, 2000). This estimation is based on a wastewater production of about 200 l per person per day.

Measurements in municipal influents are generally lower than these estimated values, for example in the Netherlands, values ranged from 20-130 ng/l for E1, from 17-150 ng/l for E2 and <0.3-5.9 ng/l for EE2 (Vethaak *et al.,* 2002). The samples were filtered first, so only the dissolved hormones were measured, and no deconjugation step was applied, although a considerable amount of conjugated estrogens can be present in influents (58% of total E1 and E2 and 26% of EE2 (Adler, 2001).

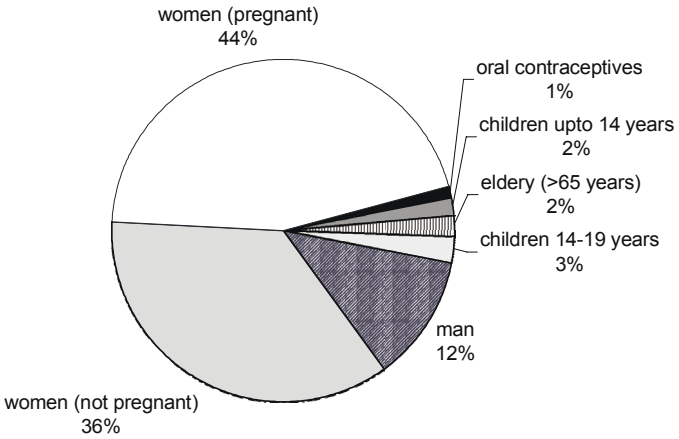


Figure 2.1. Contribution of different groups of the Dutch population to the amount of total excreted natural estrogens and 17α-ethynylestradiol; based on the total population figures of 2001 (CBS, 2002).

2.3 Environmental consequences and prevalence

Estrogens present in discharged domestic STP effluents represent the most significant input to the aquatic environment and serve as important point sources, especially in densely populated areas (Belfroid *et al.,* 1999b;Ternes *et al.,* 1999b). Figure 2.2 illustrates this pathway. Worldwide several research groups posed the hypothesis that increased estrogen concentrations in the environment can be associated with the observed adverse trends in reproductive health, and the prevalence of cancer in endocrine sensitive tissues (e.g. breast, prostate, testis) (Bosland *et al.,* 2002; USDHHS, 2002). For humans, however, such a causal relation has not been firmly established (Safe, 2000), but for fish direct correlations between the discharge of STP effluents in surface water and the feminisation of male fish was

demonstrated; early life exposure can affect the sex ratio by increasing the female phenotype (Jones *et al.,* 2000; Vethaak *et al.,* 2002).

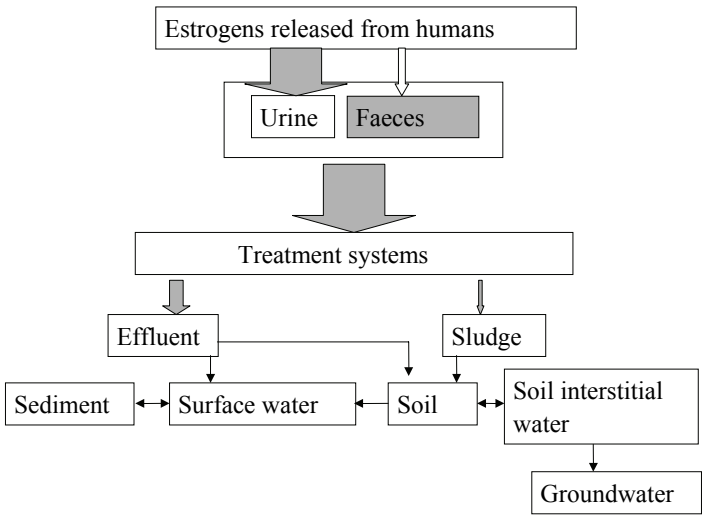


Figure 2.2. Pathway of estrogens from release by humans to ending up in the different environmental compartments.

Concentrations as low as 0.5 ng/l of EE2 induce vitellogenin production, a protein responsible for the formation of oocytes, in male trout after 10 days exposure (Purdom *et al.,* 1994; Hansen *et al.,* 1998). The predicted no effect concentration is set on 0.1 ng/l for EE2 and 1 ng/l for E2 (EA, 2002). Besides the direct effects on the aquatic environment, there is a suspected bioaccumulation via the food chain for all three compounds (Lai *et al.,* 2002; Takahashi *et al.,* 2003; Gomes *et al.,* 2004). There is even an effect observed on the plant alfalfa; irrigation with wastewater led to an elevated level of phytoestrogens (Shore *et al.,* 1995). Adding E1 and E2 to irrigation water in a concentration range of 5-500 ng/l did increase growth, while a higher concentration range 50-500 μg/l inhibited growth (Shore *et al.,* 1992).

Common median values for estrogens measured in STP effluents range from 1 to 11 ng/l for E1, E2 and EE2 in Sweden (Larsson *et al.,* 1999), Germany and Canada (Ternes *et al.,* 1999b), Soutwestern Germany (Spengler *et al.,* 2001), UK (Desbrow *et al.,* 1998), Italy (Baronti *et al.,* 2000) and the Netherlands (Belfroid *et al.,* 1999b). Values for individual measurements may be higher, the maximal value measured for E1 was 76 ng/l in the UK (Desbrow *et al.,* 1998) 64 ng/l for E2 and 42 ng/l for EE2 both measured in Canada (Ternes *et al.,* 1999b). In river water samples from Italy, values for E1, E2 and EE2 ranged from 0.04 to 1.5 ng/l (Baronti *et al.,* 2000). From a study conducted in Spain, 8.0 ng/l of E1 was detected in river water and up to 22.8 μg/kg in sediment (Petrovic *et al.,* 2002). In the UK values in river water were ranging from <0.4 to 12.2 ng/l for E1, <0.4 to 4.3 ng/l for E2 and <0.4 to 3.4 ng/l for EE2 (Williams *et al.,* 2003). Measurements done in two rivers and measured with a so called bioassay in which estrogenity is expressed as E2-equivalents (Chapter 2.4.2) gave a similar value of about 1.4 to 2.9 ng E2 equivalents/l, while measurements were near the limits of detection one kilometer upstream and downstream of the STP. Levels in the sediment were

between 21.3 and 29.9 ng E2 equivalents/kg both at upstream and downstream sites (Peck *et al.,* 2004). This suggests that river sediments are acting as a depot and potential source of estrogenic contaminants. In the Netherlands E1concentrations in surface water were detected from <0.3 to 7.2 ng/l, E2 from <0.8 to 1.0 ng/l and EE2 from <0.3 to 0.4 ng/l (Vethaak *et al.,* 2002). Estrogens were found in groundwater in Austria in concentrations up to 1.6 ng/l for E1, 0.79 for E2 and 0.94 ng/l for EE2 (Hohenbrun, *et al.* 2004). Kuch and Balsmiter (2001) have detected E1, E2 and EE2 in tap water originating from three drinking water plants in south of Germany up to 0.6, 2.1 and 0.5 ng/l respectively. E1, E2 and EE2 were not detected in tap water by other researchers (Stumpf *et al.,* 1996; Fawell *et al.,* 2001).

The fate of E1, E2 and EE2 in STPs is related to their characteristics, summarised in Table 2.1 together with their structural formulas. Many different log K_{ow} values have been reported, some of which were calculated others experimentally determined. Since in all cases the K_{ow} values are approximately 4, a considerable fraction of these compounds are likely to bind to sludge, soil and sediment. The pKa is around 10.4 for all three compounds. The solubility of EE2 is lower in wastewater than in pure water (Norpoth *et al.,* 1973). Synthetic hormones are more stable in water than natural hormones (Aherne *et al.,* 1985), which is supported by the observed ratios between EE2 and E2 in surface water, i.e. exceeding the theoretical ratio based on the ratio expected in human excreta and indicating a faster degradation of the natural estrogens (Larsson *et al.,* 1999).

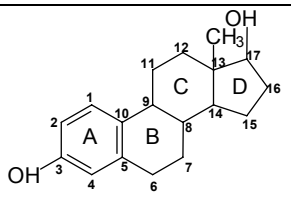
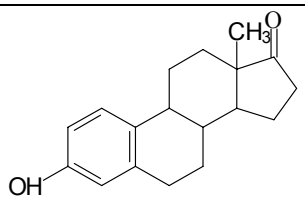
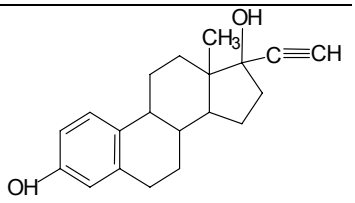
2.4 Analytical procedures

Detection and measurement of steroid estrogens in treated and raw sewage is a difficult and expensive task and far from being a routine analysis (Johnson *et al.,* 2000). As concentrations of estrogens in wastewater are generally low, a step is required to increase their concentration. In most cases, solid phase extraction (SPE) is used, either by applying SBD-XC or C18 disks or cartridges (Xiao *et al.,* 2001), but also liquid-liquid extraction (Mol *et al.,* 2000) and freeze-drying are options (López de Alda and Barcelo, 2001; Khan *et al.,* 2002; Ternes *et al.,* 2002). The latter is usually used for the analysis in non-watery matrices. For extraction, different solvents have been used; most common are methanol (Kelly 2000), mixtures of acetone/methanol (Ternes *et al.,* 2002) and acetonitrile. The sample is dried and reconstituted in methanol, acetone or acetonitrile (López de Alda and Barcelo 2000). Before or after this 'concentration step', glucuronide and sulphatase enzymes can be added to convert eventual conjugated hormones into unconjugated hormones (Belfroid *et al.,* 1999b; Huang and Sedlak 2001; Legler *et al.,* 2002). Cleaning up by a silica-column or HPLC can be applied. Latter is used to separate different hormones as they have a different residence time in the column (Belfroid *et al.,* 1999b; Huang and Sedlak, 2001; Williams *et al.,* 2003).

Concentrated samples can be analysed using different techniques such as: Gas Chromatography with Mass Spectrometry (GC/MS) (Mol *et al.,* 2000; Jeannot *et al.,* 2002), Gas Chromatography with tandem Mass Spectrometry (GC/MS/MS) (Ternes *et al.,* 2002; Williams *et al.,* 2003), Gas Chromatography Negative-Ion Chemical-Ionization Mass Spectrometry (GC/MS/NICI) (Nakamura *et al.,* 2001), Gas Chromatography with Mass Spectrometry and Electron Impact (GC/MS/EI) (Nakamura *et al.,* 2001), Gas

Chromatography Negative Chemical Ionisation Mass Spectrometry (GC/NCI/MS) (Xiao *et al.,* 2001), Liquid Chromatography with Mass Spectrometry (LC/MS) (Jeannot *et al.,* 2002), Liquid Chromatography with tandem Mass Spectrometry (LC/MS/MS) (Ingrand *et al.,* 2003; Isobe *et al.,* 2003), Liquid Chromatography Electrospray Ionisation tandem Mass Spectrometry (LC/ESI/MS/MS) (Baronti *et al.,* 2000), Liquid Chromatography with Diode Array Detector (LC-DAD) (Braga *et al.,* 2001), Liquid Chromatography with Diode Array Detector and Mass Spectrometry (LC/DAD/MS) (López de Alda and Barcelo, 2001), High Resolution Gas Chromatography with Mass Spectrometry operating in Single-Ion Monitoring (HRGC/MS/SIM) (Kuch and Ballschmiter 2000), High Performance Liquid Chromatography (HPLC) (Snyder *et al.,* 1999), immunoassay (Khan *et al.,* 2002) or bioassay (*in vitro* and *in vivo*) (Folmar *et al.,* 2002; Rutishauser *et al.,* 2004).

Table 2.1. Chemical structure and physiochemical properties of E2, E1 and EE2.

Name	17β-Estradiol (E2)	Estrone (E1)	17α-Ethynylestradiol (EE2)
Structure			
Formula	$C_{18}H_{24}O_2$	$C_{18}H_{22}O_2$	$C_{20}H_{24}O_2$
Molecular weight (g)	272.39	270.37	296.40
Aqueous solubility at 20°C (mg/l)	13[a]; 12.96[b]; 1.51±0.08[m]	13[a]; 12.42[b] ,12.4[c]; 1.30±0.08[m]	4.8[a]; 4.83[b], 3.8-4.5[d]; 4.7[e]; 9.20±0.09[m]
Henry's law constant (atm-m^3mol^{-1}; at 25 °C)	3.64E-011[f] 6.22E-012[g]	3.8E-010[f] 6.12E-012[g]	7.94E-012[f] 3.75E-012[g]
Log K_{ow}	3.94[f]; 4.01[h]; 3.10[i]	3.4[a]; 3.43[f]; 3.13[h]	4.1[a]; 4.15[f]; 3.67[h]; 3.9[i];
Log Koc	2.94[n]; 3.64[o]; 3.57[p]	2.99[n]; 3.47[p]	3.68[n]; 3.73[p]
Size (nm)[j]	0.398	0.396	0.416
pKa	10.46±0.03[k1]; 10.4[l]	10.34±0.05[k]	10.40±0.0[k]; 10.7[l]
Vapour pressure (Pa)	3.0E-8[q]	3.0E-8[q]	6.0E-9[q]

[a] in double distilled water ; Lai *et al.* (2000) [b]Tabak *et al.* (1981); [c] Jürgens *et al.* (1999); [d] In wastewater Norpoth *et al.* (1973); [e]In wastewater (Tabak *et al.,* 1970); [f]Calculated (SRC 2003); [g]Lai *et al.* (2002); [h] Hansch (1995); [i]Jürgens *et al.* (2002) and Holthaus *et al.* (2002); [j]Calculated according Worch (1993) and Stokes Einstein in Schäfer *et al.* (2003) [k]Hurwitz and Liu (1977) [k1] value for 17α-estradiol; [l]Clara *et al.* (2004b); [m]Shareef *et al.* (2006); . [n]Casey *et al.* (2003) for soil; [o]Ying *et al.* (2002) for aquifer material; [p]Ying *et al.* (2005)for soil; [q]Yu *et al.* (2004).

A derivatisation step is needed for GC analysis in order to make the compounds more volatile. For this purpose mixtures of N-methyl-N-tert.-butyldimethylsilyltrifluoracetamide (MTBSTFA) containing 1% tert.-butyldimethylchlorosilane (TBDMCS) (Kelly, 2000; Williams *et al.,* 2003), pentafluorobenzylbromide and trimethylsilimidazole (Braga *et al.,* 2001), or heptafluorobutyric anhydride (Huang and Sedlak, 2001) can be used. Further information can be obtained from a report of the Danish Environmental Protection Agency on

the evaluation of analytical chemical methods for detection of estrogens in the environment (Ingerslev and Halling-Sørensen, 2003) and in Chapter 3 of this thesis.

2.4.1 Immunoassay

Two immunoassay techniques are the enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). These techniques are based on the reaction with antibodies. Although antibodies are biologically derived reagents, immunoassays are physical assays based on the law of mass action (Bunce *et al.,* 2000). The antigen, estrogens in a sample, will bind to the inner surface of a well or tube coated with a protein antibody. A certain amount of estrogen, colouring enzyme or radioactive labelled, is added. When the sample contained a substantial amount of estrogens, less binding places are available for the labelled estrogens. The excess estrogens are rinsed out and next a substrate is added to the antibody tubes: the amount of bound antigen-enzyme conjugates develops a colour (Wako, 2002). A higher concentration of estrogens in the sample, leads to less antigen-enzyme conjugates and a lighter colour. In case of radio labelled estrogens, a scintillication technique is used. Disadvantages of this technique include cross-reaction and matrix effects (Voulvoulis and Scrimshaw, 2003).

2.4.2 Bioassay

Competitive ligand binding assays, cell proliferation assays and *in vitro* gene expression assays represent the most common *in vitro* approaches for estrogenic compounds (Snyder *et al.,* 2000). A bioassay makes use of common mechanisms of action as occurring in vertebrates. Estrogens are transported by the blood mainly bound to sex hormone binding globulins; free estrogens can exert their action by diffusing through cell membranes and binding to estrogen receptors (ER) (Legler, 2002). ERs are found in many tissues, including reproductive organs and accessory sex organs, brain, bone and liver. After dimerization of two ER-ligand complexes, binding to estrogen response elements of genes on the DNA in the nucleus takes place. After transcription, mRNA is translated into protein by ribosomes.
Competitive ligand binding assays are based on the fact that estrogen agonists and estrogen antagonists are binding to the ER and that both will lead to positive responses (Bunce *et al.,* 2000). This assay establishes the ability of compounds to bind *in vitro* to the ER, thereby displacing (radioactive) labelled E2 from the ER. The amount of radio-ligand bound in the control compared to amount radio-ligand bound in the sample leads to a quantification of the amount of estrogens present in the sample. The most serious limitation of these assays is that - although compounds may bind to the receptor the tests do not distinguish between agonistic and antagonistic effects (Zacharewski, 1997, 1998).

Cell proliferation techniques, also known as “E-screen”, are based on human-derived cell lines; they utilize a number of end points to assess the cell proliferation induced through exposure to estrogenic compounds (Voulvoulis and Scrimshaw, 2003). A major problem is the reproducibility, since many different cell lines are in use.

The *in vitro* gene expression assays, also called recombinant reportergen assays, are undertaken with genetically engineered mammalian cells or strains of yeast. The cells have been transfected or transformed with recombinant DNA, so that exposure to estrogens not

only lead to the production of the intended protein, but for example also to the production of an enzyme. In the ER-Calux method the enzyme luciferase is produced with the aid of modified human breast cancer cells (Legler *et al.,* 2002) and in the YES-assay β-galactosidase is produced by modified yeast cells (Murk *et al.,* 2002). Luciferase can be measured by adding luciferin, which will result in a light producing reaction and β-galactosidase which will change the yellow coloured assay medium (chlorophenol red-B-D-galactopyranoside) to red. The ER-calux assay appeared to be the most sensitive with a detection limit of 0.5 pM, followed by the YES assay with a detection limit of 10 pM for E2 and the ER-binding assay with 1000 pM (Murk *et al.,* 2002).

Although *in vitro* assays represent an attractive option because they are fast, inexpensive and fairly well reproducible, they may miss effects that might take place merely in whole organisms (Snyder *et al.,* 2000). This makes *in vivo* assays necessary for the evaluation of impacts on the endocrine system as a whole (Voulvoulis and Scrimshaw, 2003).

2.4.3 In Vivo

For *in vivo* experiments in the aquatic environment fish are frequently used. For example, adult male rainbow trouts and adult roaches were exposed to STP effluent levels of estrogens and after the exposure, plasma levels of vitellogenin were determined to assess the estrogenic response (Purdom *et al.,* 1994; Routledge *et al.,* 1998; Jones *et al.,* 2000; Folmar *et al.,* 2002). The presence of vitellogenin in male fish is a useful biomarker for identifying estrogenic activity in sewage effluents (Hansen *et al.,* 1998; Jones *et al.,* 2000; Solé *et al.,* 2001; Hennies *et al.,* 2003). Besides, the increased vitellogenin levels, after long-term exposure to STP effluent estrogens also had an impact on their condition and gonadal growth in roach (Rodgers-Gray *et al.,* 2000).

2.4.4 Validation

Different studies have been performed dealing with the validation of methods. It was concluded that compared to GC/MS/MS, the immunoassay (ELISA) technique has lower detection limits, requires less pre-concentration, and is less susceptible to matrix interference (Huang and Sedlak 2001). The theoretical values of estrogenic activity calculated from the concentrations of each estrogen by LC-MS/MS in treated wastewater were found to correlate well with values of estrogenic activity measured by yeast estrogen screen assay (Onda *et al.,* 2003).

Storage of samples is an important issue to be considered since severe losses of estrogens were observed during storage of bottled river water after 7 days at 4°C. The best way to store samples showed to be on the carbograph material in a cartridge (after SPE) and stored at -18°C (Baronti *et al.,* 2000). The different techniques used for determination can lead to serious difficulties in comparing results, as they have different precision and detection limits. It is also not always clear whether or not a pre-deconjugation step was applied during the measurements in different researches. Also the method of pre-concentrating samples from different matrices seems to have a large impact on the recovery of estrogens and thus on the measured amounts.

2.5 Fate in biological STPs

Processes playing a role in the removal of estrogens from the aquatic phase are: adsorption, biodegradation, and photolytic degradation. Volatisation is not expected to play a significant role in the removal of E2, E1 and EE2, since compounds with a Henry's law constant (H_c) lower than 1E-04 and an H_c/K_{ow} ratio below 1E-09 have a low volatisation potential (Rogers, 1996)(Table 2.1). These processes were linked with each other for the three estrogens in model developed by Joss *et al.* (2004), describing the behaviour in STPs, which is illustrated in Figure 2.3 and 2.4. In the figures it is assumed that estrogens need to be present in the liquid phase, in unconjugated form to be biodegraded. Under aerobic or anoxic conditions, E2 will be first oxidised to E1, which subsequently is oxidised to unknown metabolites and finally to CO_2 and water. EE2 is oxidised to unknown metabolites and ultimately to CO_2 and water. Under anaerobic conditions, theoretically E1 can be reduced to E2. More information on adsorption and degradation under different environmental conditions is outlined in Chapter 2.5.1 and 2.5.2.

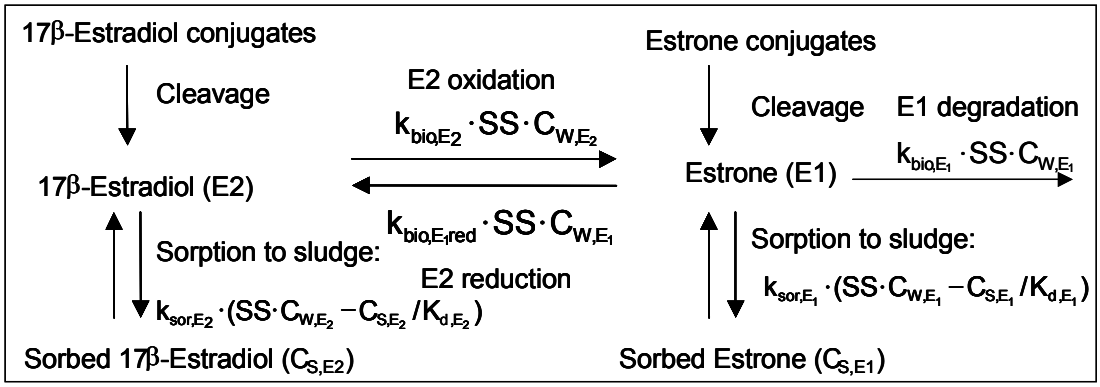


Figure 2.3. Scheme of the behaviour of estrone (E1) and 17β-estradiol (E2) in STPs, adopted from Joss *et al.* (2004), Abbreviations: k_{bio} and k_{sor} are pseudo-first-order rate constants; SS suspended solids; C_w bulk soluble concentration; C_s sorbed concentration per reactor volume; K_d sorption coefficient.

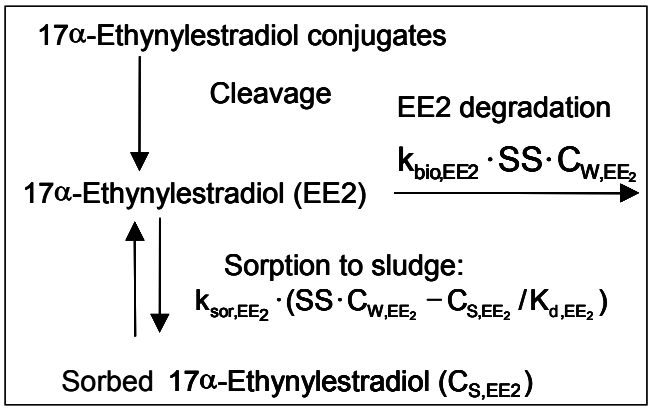


Figure 2.4. Scheme of the behaviour of 17α-ethynylestradiol (EE2) in STPs, adopted from Joss *et al.* (2004) (Abbreviations see Figure 2.3).

2.5.1 Adsorption

Adsorption to organic material can be expected to play a significant role in reducing concentrations of dissolved compounds in the aqueous phase. The octanol-water (K_{ow}) and

organic carbon (K_{oc}) partition coefficients are indicators used for the adsorption tendency to organic material. A relation between log K_{oc}, water solubility and log K_{ow} has been presented by Means et al (1982), viz. the Equations 1 and 2, in which S is the water solubility (mg/l).

$$\log K_{oc} = 0.686 \log S + 4.273 \qquad \text{Equation 1}$$

$$\log K_{oc} = \log K_{ow} - 0.317 \qquad \text{Equation 2}$$

Estrogens are hydrophobic organic compounds, with a low volatility and with log K_{ow} values of 3.43 for E1, 3.94 for E2 and 4.15 for EE2, and a water solubility at 20 °C of 13 mg/l for E1 and E2 and 4.8 mg/l for EE2 (Lai *et al.,* 2000), but as follows from Table 2.1, different values for solubility have been reported. As an indication, compounds with a log K_{ow} below 2.5 exhibit a low sorption potential, between 2.5 and 4.0 a medium sorption potential and higher than 4.0 a high sorption potential (Rogers, 1996). According to model calculations the fate of chemicals in STPs would give a 46% loss with discharged sludge for a compound with log K_{ow} 4 (Struijs *et al.,* 1991; Panter *et al.,* 1999). Applying the same model gives <10% loss with discharged sludge for a compound with log K_{ow} 3 and >75% for a compound with log K_{ow} 5. Since the reported log K_{ow} values for E1, E2 and EE2 vary between 3-4 the fraction eventually discharged with the sludge cannot be well predicted. In advance of results later in this chapter, it is shown that the above mentioned value of 46% is an overestimation; at most 5% of E1, E2 and EE2 ultimately will be discharged with the sludge.

Frequently, sorption is described using the empirical Freundlich adsorption isotherm, i.e. Equation 3. In this equation C_s represents the concentration on the sorbent (solid phase) at equilibrium, C_w the equilibrium concentration in the water phase and K_f and n_f are the sorption coefficient and constant. At very low concentrations, when the binding places are not fully occupied (n_f =1), the relation with the sorption coefficient K_d (l/kg) becomes linear (Equation 4).

$$C_s = K_f \cdot C_w^{n_f} \qquad \text{Equation 3}$$

In which:
C_s: concentration in the solid phase (mg/kg VSS)
C_w: concentration in the liquid phase (mg/l)
K_f: sorption coefficient ($l^n.mg^{1-n}.kg^{-1}$)
n_f: sorption constant (-)

$$C_s = K_d C_w \qquad \text{Equation 4}$$

Several tests were conducted to assess the adsorption behaviour of estrogens on activated sludge, anaerobic sludge, sediments, soils or other organic materials. From the research dealing with the sorption on sediments, three sorption phases were distinguished, viz. a rapid sorption during 0 and 0.5 h, followed by a period of slower sorption up to 1 h and then with a period with desorption, explained by an increase in dissolved organic matter in the water phase (Lai *et al.,* 2000). With activated carbon, an equilibrium for the sorption of E2 was

reached after 50-180 minutes (Fuerhacker *et al.,* 2001). In adsorption isotherms found by Jürgens *et al.* (1999) an 'equilibrium' with the sorption on river sediments is nearly reached after two days, although even after 5 days the adsorbed amount still was increasing. According to results of Bowman at al. (2003) a final equilibrium only established after 50 days on river water sediments. The time required for establishment of an equilibrium is clearly related to the type of sorption material and the test conditions applied. Roughly, after a period of several hours more than 90% of the equilibrium concentration is already reached.

Sorption to sediments

On sediments, for E1, K_f was 54 $l^n.mg^{1-n}.kg^{-1}$ (K_d 8 l/kg), for E2 36 $l^n.mg^{1-n}.kg^{-1}$ (K_d 4 l/kg) and for EE2 52 $l^n.mg^{1-n}.kg^{-1}$ (K_d 5 l/kg) and n_f was for E1 0.73, E2 0.67 and EE2 0.83 (Lai *et al.,* 2000). A sorption constant below 1 indicates that sorption approached a limit and the binding sites have been occupied. The limited amount of binding sites is illustrated after addition of the superhydrophobic compound estradiol valerate (K_{ow}=6.41) which showed supressed sorption of the other added estrogens, suggesting a competition for binding sites (Lai *et al.,* 2000). Sorption increased with increasing salinity by adding NaCl, leading to aggregation and flocculation. Holthaus *et al.* (2002) conducted adsorption experiments in bed sediments under anaerobic conditions in the lower concentration range and therefore the simplified isotherm (Equation 4) was used to calculate the K_d values for E2 and EE2 on different bed sediments. Around 80-90% of the equilibrium was achieved within one day, but a complete equilibrium was only achieved after two days. EE2 showed a greater affinity to the bed sediments in all cases, with sorption K_d values two to three times higher than those determined for E2. The K_d values were ranging from 4 –72 l/kg for E2 and 8-121 l/kg for EE2. In general, higher K_d values were associated with smaller particle size and higher organic carbon content in bed sediments.

Sorption to organic materials

Sorption of E2 and EE2 was investigated onto several commercially available organic colloidal compounds including polysaccharides and humic substances, which are found in typical streams and rivers (Yamamoto and Liljestrand 2003). K_{oc} values were calculated with Equation 5, in which C_w is the concentration in solution and $C_{w,0}$ the concentration in absence of organic colloids.

$$C_{w,0} / C_w = 1 + K_{oc}[Organic\ colloid\ (kgTOC / l)] \qquad \text{Equation 5}$$

Yanamoto & Liljestrand (2003) found sorption of E2 and EE2 to be highest on tannic acid, with a log K_{oc} of 5.28 and 5.22 respectively and the lowest for the polysaccharide algic acid, 2.62 and 2.53 respectively. The values for some humic acids were somewhat lower than for tannic acid. So binding is better onto substances containing aromatic rings, caused by interaction between π-electrons. The poor relationship between log K_{ow} and log K_{oc} suggests significant contributions of other sorption mechanisms than hydrophobic interaction. In a pH range of 4-9.5 at a constant ionic strength of 0.02 M and for different ionic strengths ranging

from 0.01-1 M at a constant pH of 7, no significant differences in K_{oc} were observed. It was concluded from this research that in natural waters containing 5 mg TOC/l, approximately 15 to 50% of the estrogens are bound.

Sorption to sludge

For sorption of E1 and E2 to activated sludge the highest observed percentage adsorbed was 23% at pH 8 and 55% at pH 2 (Jensen and Schäfer, 2001). At a concentration range of 5-500 ng/l radio labelled E1 and E2, adsorption to activated sludge was linear, indicating adsorption sites are in excess (Schäfer *et al.,* 2002b). The adsorption percentage is depending on the sludge concentration, as approximately 15% of E1 was adsorbed at approximately 2 g sludge/l and 30 % was adsorbed at 8 g sludge/l.

In wastewater, containing 128 mg SS/l, spiked with radio labelled E2 to a concentration of 50 ng/l, 86% of the radioactivity remained in the liquid phase after 24 hrs (Fürhacker *et al.,* 1999). This research approached the fate in sewer systems, as raw municipal wastewater was spiked, with no addition of activated sludge, and incubation was without aeration. In a test with activated sludge at a concentration of 2-5 g SS/l, only 20% of labelled EE2 remained in the aqueous phase after one hour, when 20% mineralisation was observed, concluding that 60% was bound to the sludge (Layton *et al.,* 2000). During a biological oxygen demand (BOD) test, 28% of E2 and 68% of EE2 was calculated to be sorbed to sludge after 3 hrs incubation, which is greater than 20% and therefore considered of significance (Kozak *et al.,* 2001).

Joss *et al.* (2004) has used a dynamic sorption kinetic model to calculate the flux from the soluble phase to the solid sludge particles resulting in Equation 6:

$$r = k_{sor} \cdot (SS \cdot C_{W,bulk} \cdot \frac{C_{S,reactor}}{K_d}) \qquad \text{Equation 6}$$

In which r is the flux ($g.l^{-1}.d^{-1}$), k_{sor} is the pseudo-first-order sorption rate constant ($lg\ SS^{-1}.d^{-1}$), $C_{W,bulk}$ is the soluble estrogen concentration in the bulk liquid phase (g/l), $C_{S,reactor}$ is the sorbed estrogen amount per reactor volume (g/l) and K_d is the adsorption coefficient. Using this model on the results of an STP in Wiesbaden, Germany (Andersen *et al.,* 2003), resulted in a $k_{E1,sor}$ of 4100±800 $l.kg^{-1}.d^{-1}$ and a K_{dE1} of 900±100 l/kg (Joss *et al.,* 2004). No values could be calculated for E2 and EE2, as the data were too close to their analytical limit of quantification. K_f and K_d values for de-activated sludge from the Ega STP, the Lundtofte STP and an Austrian plant are shown in Table 2.2. Sludge was de-activated by freeze-drying, followed by sterilization for 2 hours at 103°C in the first two cases. This pre-treatment might have had some effects on the sorption properties of the sludge, resulting in high standard deviations and lower obtained adsorption coefficients than the calculated value of 900 l/kg for E1. In general, K_f and K_d values are highest for sludge in comparison with sediments and other organic materials. For the Austrian plant mercury sulphate was used for de-activation, which showed to have no effect on the adsorption capacity, as the K_f and K_d values for both activated and inactivated sludge gave the same values (Clara *et al.,* 2004b). Clara *et al*. (2004b) also researched the influence of the pH on the adsorption and found that

near the pKa value (pH>9) both E2 and EE2 started to desorb as a result of increased solubility of these compounds in the dissociated form.

Table 2.2. K_f and K_d values for batch experiments with de-activated sludge from Ega and Lundtofte STP (Kjølholt *et al.,* 2004) and with activated and de-activated sludge from an Austrian STP (Clara *et al.,* 2004b). Standard deviation in brackets.

Sludge origin	Compound	K_f ($l^n.mg^{1-n}.kg^{-1}$)	K_d (l/kg)
Ega STP;de-activated by freeze-drying and sterilisation	E1	822 (918)	249
	E2	594 (281)	236
	EE2	267 (257)	436
Lundtofte STP; de-activated by freeze-drying and sterilisation	E1	89 (105)	570
	E2	1106 (627)	360
	EE2	383 (245)	459
STP in Austria; de-activated sludge (0,5 ml/l of a Hg_2SO_4, 200 g/l solution)	E2	620	692
	EE2	480	692
STP in Austria; activated sludge	EE2	480	692

The information on adsorption to anaerobic sludge is scarce. Pakert *et al.* (2003) found in batch tests with anaerobic sludge with a TSS content of 30 g/l, that 75% of E2, 85% of E1 and 90% of EE2 was adsorbed. Kunst *et al.* (2002) reported values adsorbed to anaerobic sludge during sludge treatment of 3 to 115 µg/kg TS for E2 and 3 to 330 µg/kg TS for E1. EE2 was below the limits of detection.

2.5.2 Biodegradation

Enzymatic deconjugation

Estrogen conjugates are cleaved into their active forms, as found in batch experiments using activated sludge (Ternes *et al.,* 1999a). The initial transformation of 17β-estradiol-conjugates to an estrogenically active product occurs more rapidly than degradative loss (Panter *et al.,* 1999). Deconjugation of glucuronide conjugates is expected to already take place in sewer systems, while cleavage of the sulphuric conjugates, (which need arylsulphatase for cleavage), will only happen in STPs as this demands more specialistic micro-organisms (Baronti *et al.,* 2000). This is confirmed with measurements at the STP entrance, where free estrogens and sulphated estrogens were the dominant species (D'Ascenzo *et al.,* 2003). Also in lab scale experiments with wastewater and the addition of both types of conjugates, it took approximately 3 days for the sulphate conjugates, against 7 hours for the glucuronide conjugates to reach half the initial concentration (D'Ascenzo *et al.,* 2003).

Aerobic biodegradation

In aerobic batch experiments it was shown that after 1-3 hrs, more than 95% of E2 was oxidised to E1 (Ternes *et al.,* 1999a). In the same experimental set up, EE2 appeared to be stable. Also Norpoth *et al.* (1973) found no degradation of EE2 in activated sludge after an incubation time of five days. The findings for the conversion of E2 to E1 were confirmed in

experiments with river water samples, in which E2 was converted into E1 and mineralised according first order kinetics (Jürgens *et al.,* 2002) (Equation 7).

$$C_t = C_0 e^{-k \cdot t} \qquad \text{Equation 7}$$

In which C_0 is the initial concentration (ng/l) and C_t is the concentration at time t (days) and *k* the reaction constant (d^{-1}). The half-life, $t_{1/2}$ (days) can be calculated according Equation 8.

$$t_{1/2} = \frac{ln\,2}{k} \qquad \text{Equation 8}$$

E1 can be mineralised by cleavage initiated at the A-ring (Layton *et al.,* 2000) or initiated at C-17 of ring D (Lee and Liu 2002). In the first case, the ring is cleaved by hydroxylation at C-4, after that a oxidative fission between C-4 and C-5 by a dioxygenase, from there on it can be converted into either pyridine carbolic acid, where no CO_2 is formed, or to compound I and II noted in Figure 2.5, where CO_2 is formed (Coombe *et al.,* 1966). However, D-ring cleavage is more likely since lactone has been identified as a metabolite, a suggestion for the pathway is given in Figure 2.6 (Lee and Liu 2002). Eventually, estrogens will be mineralised, as after 25 days, 24-45% of radio labelled ^{14}C E2 has been converted to CO_2 by micro-organisms from river water (Jürgens *et al.,* 2002) and 70-80% was converted into CO_2 by sludge from municipal STPs after 24 hrs (Layton *et al.,* 2000). Also EE2 can be mineralised as after 24 hrs 40% of ^{14}C-EE2 was converted into CO_2 (Layton *et al.,* 2000). All the *k*-values obtained from literature are summarised in Table 2.3. In this table, an attempt has been made to standardize *k*-values for the applied dry matter content in different batch tests and therefore expressed in l.g $SS^{-1}.d^{-1}$. Accordingly, the half-life is calculated for 1 g SS. The general trend in the conversion rates is that the conversion of E2 to E1 is rapid, in some cases even a few minutes, and that EE2 is sometimes not converted at all, or at a far slower rate, with half-lives of 6 hours up to 5 days.

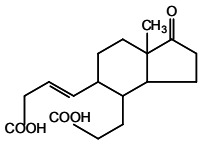
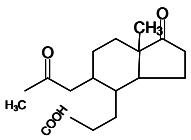
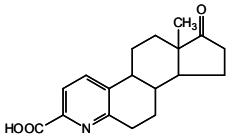
Name	I: 3aα-H-4α-[3'-propanoic acid]-5β-[2-ketopropyl]-7aβ-methyl-1-1indanone	II: 3aα-H-4α-[3'-propanoic acid]-5β-[4'-but-3-enoic acid]-7aβ-methyl-1-1indanone	pyridine carboxylic acid: 2-carboxy-7aβ-methyl-7-keto-9aαH-indano[5;4f]-5aα;10;10aβ;11-tetrahydrohydroquinoline
Structure			

Figure 2.5. Products after ring cleavage of estrone (E1) (Coombe *et al.,* 1966).

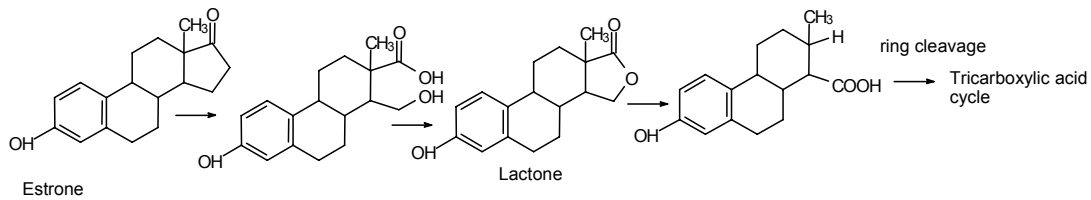


Figure 2.6. Suggested route of ring cleavage of estrone (E1)(Lee and Liu, 2002).

Table 2.3. First order degradation constants for batch tests under aerobic circumstances for E1, E2 and EE2 (SD in brackets).

References	Sludge	Feeding conditions	Temp (°C)	Compound	Initial conc.	*k*-value (1/h)	SS (g/l)	*k*-value (l.g $SS^{-1}.d^{-1}$)	$t_{1/2}$ at SS of 1 g/l	Unit
Kjøholt *et al.* (2004)	Activated sludge	Artificial wastewater 100 mg BOD/l at t=0 than 25 mg BOD/day	16	E1	500 (ng/l)	8.44	0.5	405 (158)	2.46	Minutes
				E2		16.0		768 (419)	1.3	Minutes
				E1+E2		5.26		271 (183)	3.68	Minutes
				EE2		0.063		3.03 (1.21)	5.55	Hours
				E1-3Glu		2.58		60 (65)	8.05	Minutes
				E1-3Sulf		na		0	Not degraded	
Shi *et al.* (2004a)	Activated sludge	Mineral salts medium	30	E1	1.0 mg/l	0.056 *0.047*	2.7	0.50 *0.42*	1.39 *1.66*	Days
				E2		1.3 *0.94*		11.56 *8.36*	1.44 *1.99*	Hours
				EE2		0.035 *0.022*		0.31 *0.20*	2.23 *3.54*	Days
				E1	0.2 mg/l	0.036 *0.035*	0.7	1.23 *1.20*	13.48 *13.86*	Hours
				E2		0.6 *0.34*		20.57 *11.66*	48.52 *85.62*	Minutes
				EE2		0.059 *0.039*		2.02 *1.34*	8.22 *12.44*	Hours
	Activated sludge +ATU			E1		0.004 *0.033*		0.14 *1.13*	5.05 *0.61*	Days
				E2		0.32 *0.34*		10.97 *11.66*	1.52 *1.43*	Hours
				EE2		0.0085 *0.008*		0.29 *0.26*	2.38 *2.63*	Hours
	Pure culture *Nitrosomas europea*			E1	0.4 mg/l	0.0084	n.a.		n.a.	
				E2		0.0091				
				EE2		0.0078				
	As above +ATU			E2		0				
Vader *et al.* (2000)	Activated sludge, nitrifying	Hydrazine (10 mg/l)	20	EE2	50 µg/l	0.025	1	0.6	1.16	Days
	Activated sludge, low nitrfying					0		0	Not degraded	
Joss *et al.* (2004)	Activated sludge	No feed, only fed before exp. With final effluent	16	E1	500 ng/l	2.025	0.3	162 (25)	6.16	Minutes
				E2		4.375		350 (42)	2.85	Minutes
				EE2	100 ng/l	0.1		8 (2)	2.08	Hours
	MBR sludge			E1	500 ng/l	5.1958		430 (55)	2.32	Minutes
				E2		11.4792	0.29	950 (120)	1.05	Minutes
				EE2	100 ng/l	0.0725		6 (1)	2.77	Hours
Shi *et al.* (2004b)	Activated sludge	Mineral salts medium	30	E1	20-25 mg/l	0.013	2	0.16	4.47	Days
				E2		0.0183		0.22	3.15	Days
				EE2		0		0	Not degraded	
	Night soil composting sludge			E1		0.0083		0.1	6.93	Days
				E2		0.0146		0.18	3.96	Days
				EE2		0		0	Not degraded	

Ternes *et al.*, (1999a)	Activated sludge	No Feed	n.a.	E1	1 mg/l	0.042	0.26	3.88	4.29	Hours
				E2		1.08		99.69	10.2	Minutes
				EE2		0		0	Not degraded	
	Activated sludge	No Feed	n.a.	E1	1 µg/l	0.48	0.52	22.15	45.05	Minutes
				E2		10.3		475.38	2.1	Minutes
				EE2		0		0	Not degraded	
Kikuta and Urase (2003)	Activated sludge, at pH 6.7	Artificial wastewater	20	E1	100 µg/l	0.109	2.663	0.98	16.93	Hours
				E2		6.84		61.64	16.19	Minutes
				EE2		0.013		0.12	5.92	Days
	Activated sludge, at pH 5.6			E1		0.263	2.774	2.28	7.31	Hours
				E2		8.39		72.59	13.75	Minutes
				EE2		0.105		0.91	18.31	Hours
	Activated sludge, at pH 4.4			E1		0.167	2.189	1.83	9.09	Hours
				E2		13.33		146.14	6.83	Minutes
				EE2		0.088		0.96	17.24	Hours
Fujii *et al.* (2002)	Gram negative bacterium isolated from activated sludge	Mineral salts medium	25	E2	1 g/l	0.0016	0.8E+3 to	n.a.		
					0.3 g/l	0.0024	0.5E+09			
					0.17 g/l	0.0033	CFU/ml			
Onda *et al.* (2003)	Activated sludge	No Feed	20	E1	16 µg/l	4.19[a]	2	50.28	14.19	Minutes
				E2		5.85[a]	2	70.32	8.62	Minutes
Layton *et al.* (2000)	Activated sludge	No Feed	5-10	E2	58 µg/l	0.174	2.165	1.93	10.42	Hours
				EE2	72 µg/l	0.006		0.07	5.96	Days
			22-25	E2	58 µg/l	0.252		2.79	5.21	Hours
				EE2	72 µg/l	0.012		0.13	14	Days
Lee and Liu (2002)	E2 degrading bacteria	Mineral salts medium	21	E2	200 µg/l	0.048[b]	n.a.		1.4	Hours
						0.021[c]			1.2	Days
Shi *et al.*,(2002)	Fungus *F. proliferatum*	No Feed	30	EE2	25 mg/l	0.025			14.19	Days

SS= Suspended Solids, ATU= N-Allylthiourea: chemical that inhibits nitrification, n.a.=not available.
When no values were availbale least-square method was used for calculation. For Shi *et al.* (2004a) *k*-values have been recalculated, since the reported values did not give an accurate curve-fit; recalculated figures in italic.
[a]Estrogens only measured in the supernatant; therefore, the *k*-value is an overestimation since it includes adsoption.
[b]Cyclone fermentor.
[c]Rotary shaker.

Natural estrogens are thought to be biodegraded via a pathway where bacteria can use the conversion for growth, as EE2 is thought to be biodegraded by co-metabolism, in which an organic compound is modified but not utilised for growth (Vader *et al.,* 2000).

Nitrifying sludge is held responsible for the conversion of EE2 by the use of the enzyme ammonium monooxygenase, which insert oxygen into C-H bonds (Vader *et al.,* 2000). The nitrifying activated sludge converted EE2 to more hydrophilic metabolites almost completely in about six days, while sludge with a very low nitrifying capacity did not convert EE2 (Vader *et al.,* 2000). Using N-Allylthiourea (ATU), a chemical that inhibits the nitrification by blocking the ammonium monooxygenase enzyme, resulted in slower conversion of EE2, while the conversion rates of E1 and E2 remained the same (Table 2.3). If ATU is applied on a pure culture of nitrifying bacteria the conversion is completely blocked, whereas in activated sludge it was only slowed down, suggesting that in activated sludge also other bacteria are able to convert EE2.

Another remarkable trend shown in Table 2.3 is that the conversion appears to be a lot faster when the initial concentration of the estrogens is lower. When plotting the initial concentration against standardized *k*-values there seems to be an inverse correlation, which is even more clear when plotting the applied amount of estrogens per gram SS (charts not included). This can either indicate an inhibition of the estrogens on the sludge or it can be due to another unknown phenomenon. Inhibition by EE2 has been confirmed in a biological oxygen demand (BOD) test with activated sludge at 28 °C in the dark, and with addition of 60 mg/l E2 or EE2. E2 is biodegraded, but addition of EE2 led to a lower BOD than the blank (Kozak *et al.,* 2001). The latter was confirmed in a toxicity test with nitrifying sludge, a sensitive group of micro-organisms towards toxicants, which shows toxic effects for concentrations above 10 mg EE2/l (Kozak *et al.,* 2001). This trend of higher conversion rates at lower concentrations has also been found in a river water sample, when the conversion rate for E2 was slightly higher spiking with 0.1 μg/l compared with 100 μg/l, whilst oxygen depletion was not the case (Jürgens *et al.,* 2002). Also Ternes *et al. (*1999a) observed faster degradation at 1 μg/l of E2 compared to 1 mg/l. Another example was illustrated by Shi *et al.* (2004a), finding faster degradation at 0.2 μg/l compared to 0.2 mg/l.

The degradation rate is depending on the temperature, as in a temperature range of 5-10 °C, the *k*-values are 4.2 d^{-1} for E2 and 0.14 d^{-1} for EE2, whilst in the range of 20-25 °C, the *k*-values are 6.0 d^{-1} for E2 and 0.29 d^{-1} for EE2 (Jürgens *et al.,* 2002).

Sludge origin is of importance as sludge from a STP was able to remove 84% of ^{14}C-E2 and 85% of ^{14}C-E1, against less than 4% by industrial sludge previously unexposed to estrogens (Layton *et al.,* 2000). The industrial sludge might consist of a different bacteria population that is not capable of converting E1 and E2. The sludge retention time (SRT) of the industrial sludge was quite long (17 d) and not reported for municipal sludge, but both glucose conversion rates were the same, indicating similar biological activity. Mineralization by activated sludge of ^{14}C-EE2 was 25-75-fold less; only 40% was converted in 24 hrs (Layton *et al.,* 2000). It is not clear whether this can be explained by the presence or absence of nitrifying bacteria, or that other bacteria were responsible for the conversion of estrogens. The sludge origin was also found important as illustrated by tests with both activated sludge

and sludge from a membrane bioreactor (MBR) (Joss *et al.,* 2004) (Table 2.3). MBR sludge showed a 2-3 fold faster conversion, which was explained by as well the longer sludge retention time of MBR sludge as the smaller floc size of MBR sludge resulting in a higher specific surface area, enhancing transfer into the floc. The SRT seems to be of most importance as no significant differences in removal rates were found during the degradation of EE2 in sludge from a conventional and sludge from an MBR system, both with similar high retention times (Clara *et al.,* 2004a).

There have been a few attempts to isolate a micro-organism that can specifically convert estrogens. The fungus *Fusarium proliferatum* has been isolated from cow manure and is capable of converting EE2 (Shi *et al.,* 2002). The fungus was able to remove 97% of EE2 at an initial concentration of 25 mg/l in 15 days at 30°C and gave a *k*-value of 0.6 d^{-1} at an optimum pH of 7.2 (Shi *et al.,* 2002). This resembles a half-life of 1.2 d, which is remarkably faster than measured in activated sludge. The role that fungi can play is degradation by production of enzymes, as was shown in a test with direct addition of the enzyme laccase to a solution of E1 and EE2. In three days around 40% of E1 and 75% of EE2 disappeared (Tanaka *et al.,* 2000). Fungi might also be responsible for the conversion of EE2 in STPs, since they can also be present in sludge. From an activated sludge plant, a gram-negative bacterium, possibly from the genus *Novosphingobium*, was isolated and was capable of degrading E1 and E2, but not EE2 (Fujii *et al.,* 2002). The culture was able to degrade 60% of E1 in 20 days and 40% of E2 in 14 days. The degradation of E2 was not enhanced by the addition of yeast extract or glucose. Twenty white-rot fungal strains have been screened for the removal capacity of a variety of (xeno) estrogens, including E1 and E2 (Fujita *et al.,* 2002). Removal was not established in seven of the tested strains for either E1 or E2 or both, in other strains the removal varied from 5.5% to over 99.9%.

Anaerobic biodegradation

Little results has been reported on the fate of estrogens under anaerobic conditions, and the available data are presented in Table 2.4. Bed sediment was used to examine anaerobic degradation of E2; which was fairly rapidly converted to E1 at 20°C, almost completely after an incubation of 2-days (Jürgens *et al.,* 2002). In batch experiments with activated sludge supernatant under anaerobic conditions (purged with nitrogen gas), 50% of the spiked amount of E2 was converted into E1 after 7 days (Lee and Liu, 2002). No further degradation of E1 was observed, so E1 may accumulate as a by-product. Autoclaved samples were used as sterile controls. EE2 tested under anaerobic conditions in river water samples showed no degradation over 46 days (Jürgens *et al.,* 1999). Under strict anaerobic conditions E1 is expected be reduced into E2, rather than E2 converted to E1. This pathway was demonstrated by Joss *et al.* (2004), who found a half-life of approximately 20 minutes and also a conversion of E2, with a half-life of 6 minutes in activated sludge and 2 minutes in MBR sludge. Thus, somehow under anaerobic conditions there are still electron acceptors available, like Fe^{3+} and various organic oxidative compounds, responsible for the conversion. Joss *et al.* (2004) even found conversion of EE2 in MBR sludge under anaerobic conditions with a rate of about 1.5 $l.g^{-1}.d^{-1}$, but this value is nearly the same as the degradation value derived from the blank

experiment, where no sludge was present. Overall anaerobic conditions resulted in much slower conversion rates compared to the same experiments under aerobic conditions. For example a half-life of 2.5 minutes for E1 under aerobic conditions, was 1.66 hours under anaerobic conditions (Table 2.3 and 2.4). No degradation of the three estrogens was found by Pakert *et al.* (2003) in batch tests with sludge from an anaerobic sludge digester.

Table 2.4. First order degradation constants for batch tests under anaerobic circumstances for E1, E2 and EE2

References	Sludge	Feeding conditions	Temp (°C)	Compound	Initial conc.	*k*-value (1/h)	SS (g/l)	*k*- value (l.g $SS^{-1}.d^{-1}$)	$t_{1/2}$ at SS of 1 g/l	Unit
Joss *et al.,* (2004)	Activated sludge	No feed, only fed before exp. with final effluent	16	E1	500ng/l	0.13	0.3	10 (1)	1.66	Hours
				E2		2.19		175 (10)	5.70	Minutes
				E1 red. to E2		0.65		52 (2)	19.19	Minutes
	MBR sludge			E1		0.26	0.22	28 (3)	35.65	Minutes
				E2		4.58		500 (200)	2.00	Minutes
				EE2	100 ng/l	0.014		1.5 (0.5)	11.09	Hours
				E1 red. to E2	500 ng/l	0.55		60 (15)	16.64	Minutes
Lee and Liu (2002)	E2 -degrading bacteria	Mineral salts medium	21	E2	200 ug/l	0.0024	n.a.		12	Days

Red=reduction, SS=suspended solids (SD in brackets)

Anoxic biodegradation

First order conversion rates under anoxic conditions are shown in Table 2.5. Under anoxic conditions the conversion rates lay in between those under anaerobic and aerobic conditions. For example the calculated half-life of the degradation of EE2 was 11 hours under anaerobic conditions, 2.8 hours under aerobic and 5.6 hours under anoxic conditions (Joss *et al.*, 2004) (Tables 2.3-2.5).

Table 2.5. First order degradation constants for batch tests under anoxic circumstances for E1, E2 and EE2.

References	Sludge	Feeding conditions	Temp (°C)	Compound	Initial conc.	*k*-value (1/h)	SS (g/l)	*k*-value (l.g $SS^{-1}.d^{-1}$)	$t_{1/2}$ at SS of 1 g/l	Unit
Kjøholt *et al. (*2004)	Activated sludge	Artificial wastewater 100 mg BOD/l at begin than 25 mg BOD/day	16	E1	500 ng/l	0.29	0.5	14 (5)	1.20	Hours
				E2		10.35		497 (283)	2.01	Minutes
				E1+E2		0.56		27 (21)	37.5	Minutes
				EE2		0.0035		0.17 (0.17)	4.13	Days
Joss *et al. (*2004)	Activated sludge	No feed, only fed before exp. With final effluent	16	E1	500 ng/l	0.38	0.3	30 (10)	33.27	Minutes
				E2		5.75		460 (60)	2.17	Minutes
				EE2	100 ng/l	0.015		1.2 (0.3)	13.86	Hours
	MBR sludge			E1	500 ng/l	1.29	0.27	115 (30)	8.68	Minutes
				E2		3.150		280 (50)	3.56	Minutes
				EE2	100 ng/l	0.034		3 (2)	5.55	Hours

SS=suspended solids (SD in brackets)

2.5.3 Photolytic degradation

Photolytic degradation of E2 and EE2 occurs; approximately 40% of the initial concentration was left after 144 hrs with a spectral distribution similar to natural sunlight, whilst no degradation in the dark controls was observed (Layton *et al.,* 2000). The half-life is 124 hrs for E2 and 126 hrs for EE2, so it would take at least ten days to degrade the components to half the initial concentration and is therefore slow compared to the biodegradation of E2. For EE2 it might be more significant, since the half-life for biodegradation is 17 d in rivers (Layton *et al.,* 2000). Experiments by Segmuller *et al.* (2000) to identify auto-oxidation and

photodegradation products of EE2 show a series of isomeric dimeric oxidation products, a molecule that exists of two EE2 molecules. This molecule might have lost estrogenic properties, but no information was provided on its stability in the environment.

2.6 Pilot plant and full-scale STPs

The efficiency of a STP to degrade estrogens is influenced by numerous parameters including microbial activity, sludge retention time (SRT), hydraulic retention time (HRT), temperature, and rainfall, all of which vary seasonally (Ternes, 1998). Different treatment systems may also affect microbial activity and therefore, estrogenic composition of STP effluents (Rodgers-Gray *et al.,* 2000). Conventional sewage treatment is typically a three-stage process (Metcalf and Eddy, 2003). The first stage is preliminary treatment, including removal of coarse matter and grit, primary sedimentation. The second stage comprises secondary treatment, including biological treatment like activated sludge tanks, nitrification and denitrification tanks followed by the third stage consisting of a secondary clarifier and sometimes tertiary treatment. Sludge is dealt with during separate sludge treatment.

Before entering a STP, besides deconjugation, (anaerobic) biodegradation and sorption to particulates takes place in the sewers (Johnson *et al.,* 2000), which can be significant in large catchment areas (Langford and Lester, 2003). It is not always clear if measured influent and effluent samples were determined on total estrogens including the conjugated, but in most cases only the unconjugated were determined as it was assumed most of the deconjugation has already taken place in the sewers. The detection methods used are in most cases only suitable to detect unconjugated hormones.

During primary sedimentation, the removal of hormones is mainly due to adsorption onto sludge. Secondary treatment can consist of an anaerobic pre-treatment but is usually an activated sludge system. In biological systems removal is due to adsorption, biological and chemical degradation. A long HRT allows more time for adsorption and degradation, whilst a longer SRT has influence on the biota and physical nature of floc particles, improving the sorption capacity and biodegradation (Johnson *et al.,* 2000). At a short SRT (<8 d), slow growing specific degraders can be washed out and adsorption will be the main process for removal (Jacobsen *et al.,* 1993).

Measurements in full-scale STPs are shown in Table 2.6, showing all individual values for E1, E2 and EE2 and also the removal percentages. Table 2.7 shows values for the total estrogenity. Many measurements have been conducted in the effluents of STPs, but only when information was available on both influent and effluent, the values are reported. An overall chart, Figure 2.7, shows averaged values for influent and effluent including the standard deviation amongst all measurements taken on full-scale plants so far.

The levels of estrogens found in STP effluents range from below the detection limit up to 100 ng/l (Desbrow *et al.,* 1998). STPs have the ability to remove natural steroid hormones from influents over a range of temperatures but may be less effective in removing the synthetic EE2 (Layton *et al.,* 2000). This is illustrated by Figure 2.7, showing an average removal in STPs of 62% for E1, 88% for E2 and 56% for EE2 measured in STPs. Removal of E1 shows a great variation, while E2 is often removed to a level below detection limits.

Table 2.6. Actual concentrations of E1, E2 and EE2, measured in influents and effluents of STPs.

References	Country	Location	Method of treatment	Detection method	Estrone E1 (ng/l)			17β-Estradiol E2 (ng/l)			17α-Ethynylestradiol EE2 (ng/l)		
					Influent	Effluent	% removal	Influent	Effluent	% removal	Influent	Effluent	% removal
Baronti *et al.* (2000)	Italy	Cobis	Activated sludge	LC-ESI- MS-MS	71 (35)	9.62 (5)	86 (10)	16.1 (7)	1.48 (1.02)	91 (8)	3.93 (5.14)	0.64 (0.31)	84 (23)
		Fregene			67 (18)	4.06(1.5)	94 (3)	9.2 (5)	0.92 (0.74)	90 (10)	3.39 (2.35)	0.68 (0.68)	80 (24)
		Ostia			50.6 (13)	44.62(25)	12 (54)	14.68 (7)	2.44 (1.19)	83 (11)	2.48 (1.76)	0.79 (0.32)	68 (26)
		Roma Sud			35.2 (10)	30.34 (16)	14 (52)	8.6 (2.3)	1.89 (0.94)	78 (12)	2.95 (2.08)	0.66 (0.37)	78 (20)
		Roma Est			50.4 (14)	7.66 (2.6)	85 (7)	9.3 (2)	0.75 (0.08)	92(2)	2.28 (1.57)	0.44 (0.20)	81 (16)
		Roma Nord			36.8 (8)	13.88 (15)	62(41)	11.46 (3)	0.98 (0.55)	91 (5)	2.95 (2.33)	0.48 (0.10)	84 (13)
Belfroid *et al.* (1999a)	Netherlands	STP A'dam-Westpoort (Oct)	Aeration tank	GC/MS/MS *Italic: after addition of enzyme for unconju-gation*	87; *200*	2.1;*2.1*	99	9.5;*10*	<0.6; *<0.6*	94	1.3; *1.5*	<0.3; *0.5*	67
		STP A'dam-Westpoort (Dec)			140	47	66	48	12	75	9.7	7.5	23
		STP Kralingsveer	Carousel		100	6.3	94	31	0.7	98	8.8	<0.2	98
		STP Eindhoven (Oct)	Aeration tank		*10.3* (1)	2.7; *5.4*	48	*11*	n.a.		*9.2*	<1.4; *<1.4*	84
		STP Eindhoven (Dec)			42	15	64	14	1.1	92	<0.2		
Vethaak et al (2002)		Several plants	Activated sludge	GC/MS/MS	20-130	<0.3-11		17-150	<0.8	70	<0.3-5.9	<0.3-2.6	
Bruchet et al (2002)	France	STP Esniere sur Oise	Activated sludge	GC/MS	20	8	60	10	3		2.4	1.4	42
Cargouët *et al.* (2004)		Evry	Activated sludge+(de) nitrification Upflow Biofilters	GC/MS	17.6 (0.5)	7.2 (0.8)	59 (5)	11.1(1.7)	4.5(1.4)	59 (14)	5.4(0.6)	3.1(0.6)	43 (13)
		Valenton			15.2(1.8)	6.5 (1.2)	57 (9)	17.4(1.7)	7.2(0.8)	59 (6)	7.1(0.9)	4.4(1.2)	38 (19)
		Colombes			9.6(1.5)	4.3(0.6)	55 (9)	11.6(0.6)	6.6(1.4)	43 (12)	4.9(1.0)	2.7(0.8)	45 (20)
		Achères	Activated sludge		11.2(2.3)	6.2(0.8)	45 (13)	17.1(0.6)	8.6(0.9)	50 (6)	6.8(1.4)	4.5(0.8)	34 (18)
Lee and Peart (1998)	Canada	Burlington (Dec)	Activated sludge	GC/MS	26 (1)	6 (0.5)	77 (2)	7 (0.6)	<5	>29 (6)	n.a.		
		Burlington (Jan)			53 (4)	8 (1)	85 (2)	14 (2)		>64 (5)			
		Dundas			69 (1)	9 (1)	87 (1)	7 (0.7)		>29 (7)			
		Edmonton			109 (5)	72 (2)	34 (4)	<5		na			
		Guelph (Dec)			66.5 (12)	17(1)	74 (5)	<5					
		Guelph (Jan)			41 (4)	14 (1)	66	15 (2)		67 (4)			

Nasu *et al.*, (2001)	Japan	27 plants	Activated sludge	ELISA	n.a.			0.03-0.090	n.d.-0.043	69			
		Autumn						0.042 med.	0.013 med.				
		Winter						0.02-0.094	0.0003-	70			
								0.047 med.	0.014 med.				
Petrovic *et al.* (2002)	Spain	Calaf			<2.5-115	<2.5-8.1	n.a.	11.0-30.4	<5.0	n.a.	n.a.	<5.0	n.a.
		Igualada			<2.5-4.6	<2.5-2.7		<5.0	<5.0-7.6				
		Piera			<2.5-13.1	<2.5		<5.0	<5.0				
		Manresa			<2.5-56.5	<2.5-7.2		<5.0-14.5	<5.0				
Shore et al (1993)	Israel	Tel Aviv	1)anaerobic tank	E2 Radio-Immuno-assay	48-141	7-39	22-64	n.a.					
			2)activated sludge		7-39	6-50							
Tabak *et al.* (1981)	USA	Batavia	Trickling filter	TLC and GLC after hydrolysis and liquid /liquid extraction	20 (20)	10 (7)	50 (61)	10	<10		1070 (810)	670 (590)	37 (73)
		Fairfield	Activated sludge		10 (7)	10	0 (70)	10	<10		890 (630)	530 (430)	40 (64)
		Lebanon			20 (14)	10	50 (35)	10	<10		880 (520)	540 (370)	39 (56)
		Milford	Trickling filter		40 (20)	20 (14)	50 (43)	10 (7)	10		1330 (610)	910 (630)	32 (57)
		New Richmond	Contact stabilisation		40 (20)	20 (7)	50 (31)	20(7)	10	50 (18)	1290 (710)	920 (580)	29 (60)
		Bethel	Trickling filter		30 (20)	20 (14)	33 (64)	10 (7)	10		1000 (640)	660 (470)	34 (63)
		Loveland			30 (20)	10 (7)	67 (32)	10 (7)	10		990 (520)	630 (400)	36 (52)
		Little Miami	Primary		50 (30)	30 (14)	40 (46)	20 (7)	10 (7)	50 (39)	1770 (710)	1320	25 (47)
		Bromely	Contact stabilisation		40 (14)	10 (14)	75 (36)	10 (7)	10		1480 (640)	1040	30 (47)
		Muddy Creek	Primary		50 (20)	30 (14)	40 (37)	20 (7)	10	50 (18)	1590 (750)	1160 (660)	27 (54)
		Hamilton	Activated sludge		20 (14)	10 (7)	50 (50)	10	<10		1270 (400)	780 (320)	39 (32)
		Glendale	Trickling filter		30 (20)	20(7)	33 (50)	10 (7)	10		1000 (690)	600 (490)	40 (64)
Adler (2001)	Germany	Southern and middle Germany	Five STPs	GCMS, Unconjugated	<0.5-20	<0.1-18	87	<0.5-4	<0.05-0.6	70	1-14	<0.05-0.6	98
					2 med.	0.3 med.		2 med.	0.2 med.		12 med.	0.2 med.	
				After adding enzymes	2-16	<0.1-57		1-22	0.2-2		1-45	<0.1-2	
					3 med.	0.4 med.		3 med.	0.9 med.		26 med.	0.4 med.	

Table 2.6. Continued

References	Country	Location	Method of treatment	Detection method	Estrone E1 (ng /l)			17β-Estradiol E2 (ng/l)			17α-Ethynylestradiol EE2 (ng/l)		
					Influent	Effluent	% removal	Influent	Effluent	% removal	Influent	Effluent	% removal
Adler (2001)	Germany	Southern and middle Germany	Five STPs	GCMS, Unconjugated	<0.5-20	<0.1-18	87	<0.5-4	<0.05-0.6	70	1-14	<0.05-0.6	98
					2 med.	0.3 med.		2 med.	0.2 med.		12 med.	0.2 med.	
				After adding enzymes	2-16	<0.1-57		1-22	0.2-2		1-45	<0.1-2	
					3 med.	0.4 med.		3 med.	0.9 med.		26 med.	0.4 med.	
			Seven STPs	GCMS, Unconjugated	2-25	<0.05-130	14	1-9	<0.05-4	30	1-9	<0.1-4	71
					6 med.	3 med.		1 med.	0.7 med.		4 med.	0.5 med.	
				After adding enzymes	8-25	<0.1-170		2-19	0.2-6		5-15	<0.1-40	
					14 med.	12 med.		3 med.	3 med.		7 med.	2 med.	
Andersen *et al.* (2003)	Germany	Wiesbaden	1) primary clarifier	GCMS	65.7	74.9	-14	15.8	10.9	31	8.2	5.2	37
			2) denitrification-1		74.9	37.3	50	10.9	10.3	6	5.2	1.5	71
			3) denitrification-2		37.3	2.8	92	10.3	<1	90	1.5	1.2	20
			4) nitrification		2.8	<1	64	<1	1.8	-80	1.2	<1	>20
			5) secondary clarifier		<1	<1		1.8	<1		<1	<1	
			Total removal				>98			>94			>88
			2) denitrification-1	GCMS On sludge (ng/g)	10.1			2.7			1.9		
			3) denitrification-2		6.9			2.3			2.2		
			4) nitrification		5.6			2.2			<1.5		
			6) digested sludge (solid)		25.2			5.1			<1.5		
			6) digested sludge (liquid)		67.1 (ng/l)			5.4 (ng/l)			<1 (ng/l)		
Schullerer *et al.* (2002)		Karslruhe	1)activated sludge with N and P removal	GCMS	130	26	80	32	3.2	90	55	9	84
			2)trickling filter		26	11	58	3.2	2.7	16	9	7	22
							92			92			87
		Ebingen	1) primary clarifier		120	65	46	35	17	51	20	n.d.	>91
			2) activated sludge with N and P removal		65	2.1	97	17	6	65	n.d.	2.7	
					2.1	n.d.		6	n.d.		2.7	1.8	33
		Lautlingen	3) activated carbon				>99			>97			91
			1) primary clarifier		49	34	31	31	29	6	59	55	7
			2) activated sludge with N and P removal		34	3.3	90	29	1.3	96	55	3.7	93
					3.3	n.d.		1.3	n.d.		3.7	2.2	41
			3) activated carbon				>98			>97			96

med.=median value, n.a.= not available
SD in brackets if available
Standard deviations for the removal are calculated according propagation of uncertainty (Rubinson 2000)

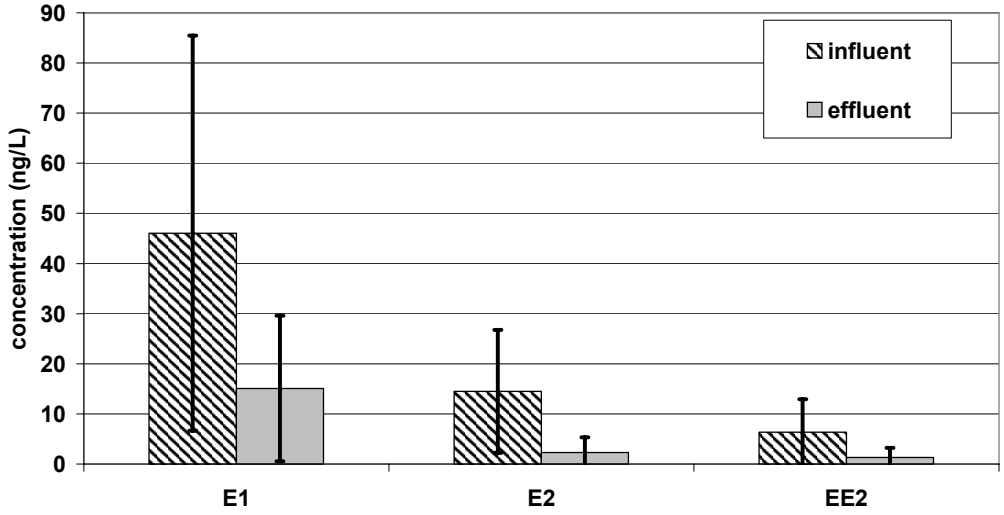


Figure 2.7. Averaged concentrations in influent and effluent over all researched STPs including error bars for the standard deviations, based on Table 2.6.

Removal of E1 varies from 10% as measured in Germany (Ternes *et al.,* 1999b) to 98% as measured in the Netherlands (Belfroid *et al.,* 1999a) and also tends to vary within the same STP. The type of treatment as well as the process conditions of each particular treatment may cause the large variation in removal. The large standard deviation for E1 in effluents of some STPs might indicate that mineralisation is not always achieved.

From Table 2.6, removal percentages for estrogens are calculated for the different processes in the treatment chain. The major reduction occurs in almost all cases during biological secondary treatment. The actual removal rates will be a reflection of the ability of the indigenous micro-organisms to biodegrade the compounds within a hydraulic residence time of approximately 14 hrs, and also of the ability of the floc particles to bind the compounds (Johnson *et al.,* 2000). The fate of estrogens along the treatment chain, as shown in Figure 2.8, will be discussed below. All values discussed can be found in Table 2.6 and 2.7.

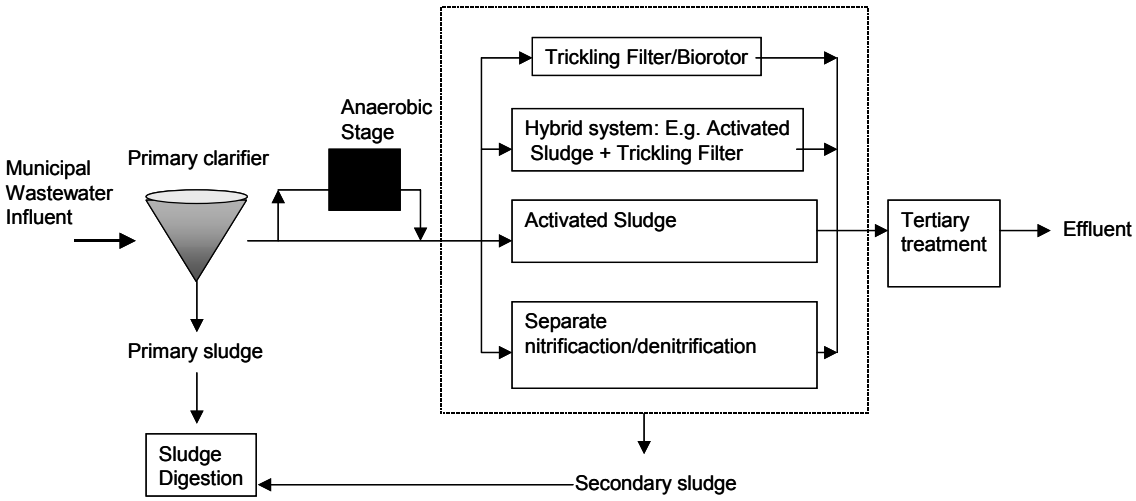


Figure 2.8. Schematic overview of the treatment chain and the different processes that are described for estrogen removal.

Table 2.7. Influent, effluent concentrations of total estrogenity

References	Country	Location	Treatment	Compound detection	Influent (ng/l)	Effluent (ng/l)	HRT/SRT	%removal per stage	% tot. removal whole plant
Svenson *et al.* (2003)	Sweden	Bureå	Precipitation; Al	Tot. estr. in equivalent E2 YES	11.90 (1.27)	12.40 (1.41)	NR	-4%	
		Järpen	Precipitation; Al		10.80 (0.14)	12.70 (1.70)		-18%	
		Sörsjön	Precipitation; Fe(III)		5.45 (0.21)	5.9 (0.30)		-8%	
		Skulsjön	Precipitation; lime		4.15 (0.78)	1.1		73%	
		Enköping	Activated sludge		29.8	12.3 (0.28)	2-8 hrs/NR	59%	
		Kattastrand	Activated sludge		5.00 (0.57)	0.3 (0.28)		94%	
		Sollebrunn	Activated sludge		10.20 (0.14)	4.3 (0.14)		58%	
		Reffelmansverket	Activated sludge		5.00 (0.71)	1.6 (0.28)		68%	
		Ljusne	Activated sludge		6.05 (0.21)	1.2		80%	
		Nolhaga	Trickling filter		22.35 (2.47)	14.85 (2.19)		34%	
		Gårlången	Trickling filter		3.05 (0.21)	10.75 (0.92)		-252%	
		Hassela	Biorotor		1.60 (0.71)	5.25 (0.21)		-228%	
		Sundet	Activated sludge		8.00 (0.42)	2.55 (0.35)		68%	
		Uddebo	Trickling filter		6.75 (0.21)	1.7		75%	
		Vimmerby	Tricklingfilter/activated sludge		6.95 (1.2)	0.1	12hrs/NR	99%	
		Kungsängsverket	Activated sludge		12.50 (0.57)	1.45	NR	88%	
		Kävlinge	Activated sludge+nitrogen		3.85(1.48)	<0.1	20hrs/NR	>74%	
		Ekebyverket	Activated sludge + wetland		19.5(2.26)	<0.1	7 d/NR	>99%	
Takigami *et al.* (2000)	Japan	Shiga night soil plant	1)Influent (mix nightsoil septage)	Tot. estr. In equivalent E2 YES[1] and ELISA for E22 in the liquid phase	4000^{1}-650^{2}		15 d/NR		>99.9%; 99.9%
			2) Denitrifiation-1			202^{1}-43.8^{2}			
			3) Nitrification		202^{1}-43.8^{2}	123^{1}-29.3^{2}		97%; 96%	
			4) Denitrifiation-2		123^{1}-29.3^{2}	43.4^{1}-18.1^{2}		39%; 33%	
			5) Sedimentation tank		43.4^{1}-18.1^{2}	6.9^{1}-11.1^{2}		65%; 38%	
			6) Flocculation		6.9^{1}-11.1^{2}	7.47^{1}-8.88^{2}		84%; 39%	
			7) Ozonation		7.47^{1}-8.88^{2}	2.2^{1}-1.41^{2}		-8%; 20%	
			8) Sand filtration		2.2^{1}-1.41^{2}	$n.d.^{1}$-0.824^{2}		71%; 84%	
			9) Activated carbon		$n.d.^{1}$-0.824^{2}	$n.d.^{1}$-0.124		-; 41%	
			10) Dewatering filtrate		5.11^{1}-4.23^{2}			-; 85%	
			1) Night soil	As above but in the sludge phase (ng/g dry matter)	624^{1}-303^{2}				
			1)Septic tank sludge		1120^{1}-274^{2}				
			2) Denitrifiation-1		305^{1}- 102^{2}				
			3) Nitrification		159^{1}- 101^{2}				
			4) Denitrifiation-2		274^{1}-107^{2}				
			5) Concentrated sludge		322^{1}-101^{2}				

Matsui *et al.* (2000)[a]	Japan	Shiga	1) Primary sedimentation	Tot. estr. In equivalent E2 YES[1] and ELISA for E2[2]	150[1] - 50[2]	140[1] -70[2]	NR	7%; -40%	93%; 80%
			2) Denitrification		140[1] -70[2]	10[1]-20[2]		93%; 71	
			3) Nitrification		10[1]-20[2]	10[1]-15[2]		0%; 25%	
			4) Secondary sedimentation		10[1]-15[2]	10[1]-10[2]		0%; 33%	
			5) Chlorination/sandfilter		10[1]-10[2]	10[1]-10[2]		0%; 0%	
			6) Dewatering filtrate		470[1] - 140[2]				
Bolz *et al.* (2000)	Germany	KA1	1) Primary clarifier	Tot. estr. In equivalent E2 (E-screen)	13	2.4	NR	82%	99%
			2) Activated sludge with N and P removal		2.4	1.5		38%	
			3) Activated carbon		1.5	0.36		76%	
			4) Secondary clarifier		0.36	0.12		67%	
			5) Final effluent		0.12	0.19		-58%	
		KA2	1) Primary clarifier		82	65		21%	99%
			2) Activated sludge with N and P removal and		65	nd			
			3) Final effluent		nd	0.74			
		KA3	1) Primary clarifier		22	17		23%	93%
			2) Trickling filter		17	2		88%	
			3) Activated sludge nitrification and P removal		2	6.4		-120%	
			4) Final effluent		6.4	1.6		75%	
		KA4	Activated sludge with N and P		16	1.3		92%	92%
		KA5	1) Primary clarifier		38	32		16%	95%
			2) Activated sludge with N and P removal		32	1.9		94%	
		KA6	Tricklingfilter with N and P removal		46	2.6		94%	94%
Kirk *et al.* (2002)[a]		WWTW A	Primary treatment	Tot. estr. In equivalent E2 YES [1]data April; May [2] data August	43[1]; 17[2]	40[1]; 14[2]	2-6h/rs/NR		7%; 18%
		WWTWB	1) Secondary treatment		75[1]; 22[2]	10[1];2[2]	4hrs/NR	87%; 91%	89%; 68%
			2) Pre UV treatment		10[1]; 2[2]	7[1]; 3[2]		30%; -50%	
			3) Final effluent		7[1]; 3 [2]	8[1]; 7[2]		-14%; -133%	
		WWTW C	1) Primary treatment		38[1]; 10[2]	30[1]; 23[2]	13.5hrs/NR	21%; -130%	>97%; 80%
			2) Secondary treatment		30[1]; 23[2]	5[1]; 2[2]		83%; 91%	
			3) Biofilter		2[1];2[2]	nd[1]; 2[2]		-; 0%	
		WWTW D	1) Primary treatment		33[1]; 27[2]	13[1]; 14[2]	13hrs/NR	61%; 48%	>97%; 96%
			2) Final effluent		13[1]; 14[2]	nd[1]; 1[2]		>99%; 93%	
		WWTW E	1) Primary treatment		77[1]; 40[2]	27[1]; 17[2]	13hrs/NR	65%; 58%	>98%; 83%
			2) Slag filtration		27[1]; 17[2]	13[1]; 13[2]		52%; 24%	
			3) Plastic filtration		27[1]; 17[2]	13[1]; 12[2]		52%; 29%	
			4) Activated sludge		27[1]; 17[2]	nd[1]; 7[2]		>99%; 59%	

SD in brackets, NR= not reported, n.d. = not detected.
[a]Values obtained from a chart

2.6.1 Primary clarifier

The initial estrogenity of the wastewater sometimes rises after passing the primary clarifier, which may indicate that deconjugation is not always complete when the wastewater enters the STPs (Kirk *et al.,* 2002). In a plant located at Wiesbaden (Germany), E1, E2 and EE2 are individually analysed and show a negative removal for E1 and positive for both E2 and EE2, presumably due to conversion of E2 into E1 and the better sorption capacities of EE2. It is highly unlikely that the latter is degraded under the anaerobic conditions in a clarifier. Addition of precipitates during primary treatment such as aluminium or iron does not increase the removal, with the exception of lime with which an unexpected removal of 73% was accomplished. In case of lime addition the pH was 11.4, which is above the isoelectric point, the estrogens were in the dissociated form, whereas with the other precipitates the pH was between 6.3 and 7.3 (Svenson *et al.,* 2003). Clara *et al.* (2004b) found that using lime in batch adsorption experiments, less E2 and EE2 was sorbed due to increased solubility, so from this point of view, no increase in removal is expected using lime. However, for a pH above 12 there seems to be a drop in concentration for EE2, so at this point some form of coagulation for this particular compound could occur. Another explanation could be that the high pH had a negative impact on the recovery of the analytical determination.

2.6.2 Anaerobic stage

The reaction fluid of an anaerobic tank in Tel Aviv, Israel was analysed for E2. The removal was 72-85%, which is in line with the expectations, as E2 can be converted to E1, also under anaerobic conditions, and a part can have been sorbed to the sludge.

2.6.3 Activated sludge

Measurements performed inside the aerated phase of the activated sludge system in the STP of Zittau, Germany and Tel Aviv, show a negative removal value (data not shown). It seems that a lot of the estrogens are desorbed. Often the slow deconjugation of the sulphate-conjugates is used as explanation, but the increase is higher than expected on base of the amounts of (sulphate) conjugates present in the influent.

On average, 57% of total E1, 67 % of total E2 and 43% of total EE2 were conjugated in raw sewage as measured in seven STPs in South and Central Germany. During treatment only a small amount is cleaved and the total amount of conjugates in the effluent remained 45% (Adler 2001). This is explained by the lack of arylsuphatase, which may be plausible as E1 is mainly excreted as sulphate conjugates and most of E1 is still present in a conjugated form in the effluent (Baronti *et al.,* 2000). Another explanation for the increased level of estrogens in the aeration tank is desorption of estrogens from sludge during the analysis, since it did not become clear whether the total sample was analysed or only the liquid phase. In Zittau, Germany, the effluent of the activated sludge process contains a higher amount of EE2 than the influent, which may be a combination of deconjugation and low biodegradability of the latter compound. During the aerobic phase of activated sludge treatment, the removal is fairly good, although sometimes a concentration over 10 ng/l is still present in the effluent. Removal percentages show a high variation between different STPs. The occurrence of

nitrification in an activated sludge system seems to have a positive effect on the removal of hormones. For nitrification a longer SRT is required because the autotrophic bacteria involved grow very slowly.

2.6.4 Trickling filter/biorotor

Removal percentages for estrogens are quite variable in a trickling filter or biorotor as can be seen in Table 2.6. They are even negative, which might be due to the relatively short hydraulic retention times in such systems, which are in the range of several minutes for high rate trickling filters and 2 hrs for low rate tricking filters (Metcalf and Eddy, 2003), so the available time for adsorption or biodegradation is short.

2.6.5 Hybrid techniques

From hybrid techniques, in which an activated sludge tank is followed by another treatment step like a trickling filter, biorotor or wetland, the activated sludge in combination with a wetland shows by far the best removal for estrogens (over 99%). Retention times in wetlands are in general quite long, which explains this high removal percentage. In this case the retention time was 7 days whilst the retention time in conventional activated sludge systems is typically 14 hours (Svenson *et al.,* 2003).

2.6.6 Differences in nitrification andenitrification tanks

Tables 2.6 and 2.7 show that the highest percentage removal of estrogens from the liquid phase is obtained during the denitrification step. Even when a second denitrification step is applied, more is removed during denitrification compared to nitrification, so it is not just due to higher concentrations in the influent or solely to a dilution of primary effluent by the return sludge and internal recirculation with low estrogen concentrations. On one occasion the concentration of E2 is increasing during the nitrification step. As this is the last step in the treatment chain, it is highly unlikely that this is due to deconjugation, though it can not be excluded; as mentioned previously, still around 40% can be present in the conjugated form in the final effluent. Perhaps the bonding of E2 onto sludge is not as strong as for the other two estrogens. In this same system, the removal of E2 in the first denitrification tank, which is receiving a nitrified stream from the nitrification tank, is also quite low, 6%, whilst the removal of EE2 is remarkably high, 71%. In the second tank, the removal of E2 is 90%. It appears that anoxic conditions enhance the adsorption of E1, E2 and EE2, since conversion rates were found to be lower in batch test under anoxic conditions compared to aerobic conditions, as described earlier.

2.6.7 Tertiary treatment

In general secondary sedimentation does not lead to an additional removal of estrogens, except for the activated sludge system treating night soil/septic tank sludge (night soil= human physiological waste collected in the morning). This significant removal could be explained by settling of colloidal material, that is formed during the treatment of this type of influents, on which estrogens are sorbed.

Other tertiary treatment systems like chlorination, sand filter, flocculation and biofilter did not show to significantly contribute to additional removal of estrogens, except for systems using activated carbon or ozonation, both able to remove over 80%. UV treatment is leading to a negative removal of estrogens measured in the liquid phase. This can be explained by the release of estrogens by destruction of sludge particles under influence of UV, which sets the estrogens free.

2.6.8 Discharge of estrogens with excess sludge

On some occasions, measurements of estrogens have been carried out in the sludge itself. The highest concentration of total estrogens adsorbed to sludge was measured in the influent of the night soil/septic tank sludge treatment plant which was approximately 1120 ng E2 equivalents/g dry matter for septic tank sludge compared to 624 ng/g dry matter in raw night soil, the same tendency was found measuring E2, specifically with ELISA, which gave 303 and 274 ng E2/g dry matter for septic tank sludge and night soil respectively (Takigami *et al.,* 2000). Apparently, these values are high as conjugated hormones may have been converted into unconjugated active estrogens during their previous stay in the septic tank, whilst the actual degradation of these compounds is absent or very low. In the activated sludge samples from the same night soil/septic tank sludge treatment system, the E2 equivalents ranged form 159-322 ng/g dry matter, with 100 ng/g due to E2. In the activated sludge tank, 50% of the total present E1 is bound to sludge, 20 % of the E2, and 60% of the EE2 (Schwarze-Scharfenberg *et al.,* 2003).

Least estrogens are bound to sludge inside nitrification tanks, 10 ng/g dry matter, which is in line with the hypothesis that during nitrification estrogens might desorb. Desorption is also occurring during sludge treatment, as estrogen measurements in the dewatering filtrates are rather high, 67.1 ng/l for E1, 5.4 ng/l for E2, and EE2 was under the detection limit (Andersen *et al.,* 2003). This phenomenon is supported by Kunst *et al.* (2002).

From the total amount of estrogens in the influent, only a very small proportion is discharged with the excess sludge from a treatment plant. In the plant in Wiesbaden (Germany), the total removal in the liquid phase is over 99% for E1 and E2, whilst only 4% of the incoming E1 and E2 is removed with the excess sludge. For EE2, over 88% is removed from the liquid phase and 5% of the incoming EE2 is removed with excess sludge. In a pilot plant, consisting of a 2.5 m^3 activated sludge tank and a settler treating pre-settled domestic wastewater, the average removal measured over one year for E1 and E2 from the liquid phase was 70 % and 95% and approximately 3% of E1 and 1% of E2 left the plant with sludge discharge (Onda *et al.,* 2003). When assuming an aeration tank is a completely mixed system, in which estrogen concentrations in sludge are similar as onto the excess sludge, it was calculated that 85% of the total amount of E1 and 95% of E2 in the tank is associated with sludge. This highlights that although the amount of estrogens removed with sludge discharge is only a fraction of the amount entering a STP, adsorption to sludge within a plant contributes to a large extent to retaining estrogens inside the STP, so that the retention is long enough for biological degradation. An exception is the Shiga activated sludge treatment system, treating nightsoil/septic tank sludge in Japan, where 99% of the estrogens are removed from the liquid

phase during treatment, and 30% of the incoming estrogens are discharged with excess sludge (Takigami *et al.,* 2000).

2.6.9 Parameters influencing removal

It is difficult to actually compare the different parameters using the available data set of full-scale plants as treatment conditions at studied STPs are often not fully described. Also different sampling strategies and methods of analysis have been used resulting in different conclusions. Also, batch experiments can not directly be translated to full-scale plants as they are often spiked with a high concentration, which may enhance the development of an adapted microbial population that would not develop under normal conditions and complicates interpretations to full-scale STPs (Snyder *et al.,* 2001). Also batch tests are likely to overestimate true biodegradation rates, as they are carried out under ideal circumstances. Alternatively, the high estrogen concentrations in batch experiments might have a toxic effect on the sludge, especially as nitrifying bacteria are very sensitive, and therefore in some of the experiments inhibition might have occurred, resulting in a lower degradation value as would be found under full-scale conditions, where the estrogen concentration is in the ng/l range. This inhibition is supported by the results of different researchers who always found a higher *k*-value in case of lower estrogen concentrations, however inhibition was not directly concluded. Most of the experiments were carried out at 20°C, whereas field conditions will be more frequently in the 10-15°C range (Johnson and Sumpter, 2001). Also in batch experiments, there is no oxygen depletion, while in activated sludge systems, anaerobic areas can appear.

Parameters influencing the degradation can be divided into parameters connected to climate, like rainfall and temperature and parameters connected to the design of a STP, like the HRT, SRT and the type of system, including the different treatment steps applied. As the latter has been discussed in detail in the previous chapter, the remaining parameters will be discussed below.

Parameters related to climate

The influence of rainfall is shown by Shore *et al.* (1993), where the concentrations of natural estrogens in the effluent were 7.5–fold higher in a dry year compared with a wet year, and removal percentages were ranging from 20-64% in the dry year to 88% in the wet year. This was also observed between samples collected in August, when the amount of rain has been substantial, compared to samples collected in May and April (Kirk *et al.,* 2002). Apparently the higher influent concentration had a larger impact on the removal percentage than the increased HRT, which was due to the reduced volume of wastewater entering the STP in dry years. Also, the longer retention time in the sewer could have enhanced the degree of deconjugation in dry periods. However due to insufficient information about the exact cirumstances in which the samples were taken, no definite conclusions can be drawn.

During winter, higher effluent concentrations for both natural and synthetic estrogens have been observed (Desbrow *et al.,* 1998; Belfroid *et al.,* 1999a; Tabak *et al.,* 1981). This can be due to a temperature effect, since biomass is less active at lower temperatures. In case

slow growing specified bacteria are playing a role in removing estrogens, especially for EE2, the reduced removal in winter can also be related to the reduced amount of these type of micro-organisms due to wash-out in winter-periods. A coarse idea on the influence of temperature on the degradation of estrogens could be obtained in an activated sludge treatment plant for municipal wastewater in both Germany and Brazil (Ternes *et al.*, 1999b) and six activated treatment plants near Rome in Italy (Baronti *et al.,* 2000). The average air temperature was 20°C for Brazil and -2°C for the German plant, the temperature of the Italian plants is not known, but presumably in between the Brazilian and German temperatures, concluding from the periods, which are October to March for Italy, excluding January and November for Germany. The samples from Brazil and Germany are flow proportional composite samples, the values from Italy are calculated with grab samples, but obtained as average of five samples at each of six different plants. The removal percentages were in order of increasing temperature, so first German, and then Italy followed by Brazil, and amounted to 14%, 59% and 83% for E1, 64%, 87 % and 99.9 for E2 and -50%, 80 % and 78% for EE2. Only the hydraulic retention times for the plants in Italy are known, which are 12-14 hours, it can only be assumed that the HRT of the other plants are in the same order of magnitude. No information is available on the sludge retention times which also may have a significance impact on the removal efficiencies. With the available data so far no correlation between the temperature and the E2 removal in full-scale treatment plants has been found (Johnson *et al.,* 2000).

Parameters related to design

Longer hydraulic retention times give higher removal efficiencies of E1, E2 and EE2 as illustrated by STPs in the UK, where removal is significantly better at an HRT of around 13 hrs compared to 2-5 hrs (Kirk *et al.,* 2002). This is confirmed by Svenson *et al. (*2003), reporting removal till below the detection limit for the Kävlinge plant with an HRT of 20 hrs and the Ekebyverket plant including a wetland with an HRT of 7 d (see Table 2.7). Approximately 99% removal was achieved in the Vimmerby plant with an HRT of 12h, which was longer than the 2-8 hrs applied in most other plants in this research, and where only about 58-94% was removed. Cargouët *et al. (*2004) found better removal for E1 (58%) and E2 (60%) in the plants Evry and Valenton with an HRT of 10-14 hrs compared to a plant in Achères with an HRT of 2-3 hrs in which a removal of 44% for E1 and 49% of E2 was achieved. In the plant containing 3 biofilters including nitrification and denitrification in Colombes with an HRT of 2.5-4 hrs, 55% of E1 and 43% of E2 were removed. In all the four plants removal for EE2 was approximately 40%.

The influence of increased SRT is illustrated by a STP in Wiesbaden which has been upgraded from a BOD removal plant to a nutrient removing plant, with substantial higher sludge retention times, increasing from <4 d to 11-13 d. Batch experiments with sludge from the old plant did not show any reduction of EE2 (Ternes *et al.,* 1999a), whereas at the increased SRT a reduction of around 90% is achieved in the full-scale plant, which can indicate the presence of micro-organisms capable of degrading EE2 (Andersen *et al.,* 2003). So below a certain SRT, degradation of EE2 will not occur in an activated sludge system.

Joss *et al.* (2004) stated the hypothesis that sludge loading is a key parameter influencing the removal of estrogens, as they found a lower degradation in the first compartments of monitored reactors. This means that micro-organisms would prefer to degrade other organic compounds over estrogens. However, no clear correlation can be found within one STP with different organic loadings in relation to the removal of estrogens. Johnson *et al.* (2000) tried to find a correlation between the flow per head and the E2 removal. Using the data from Svenson *et al.* (2003) to find a correlation between the percentage of the yearly mean flow during the time measured, a trend can be observed showing a decrease in total estrogen removal with increasing percentage of flow, indicating higher loading, but also a shorter HRT.

2.7 Fate in advanced/tertiary treatment

2.7.1 Coagulation and activated carbon

As has been observed at full-scale treatment plants, coagulation did not have any enhancing effect on the removal of estrogens, which was also tested in batch-tests by the addition of ferric chloride (5-30 mg/l) to a 15 ng/l E1 solution at different pH values (5-9) leading to no removal of E1 (Ong *et al.,* 2001). Also adsorption by iron phosphate precipitates would be unlikely to sorb large quantities of steroid estrogens (Johnson *et al.,* 2000).

Powdered activated carbon (PAC) has an adsorption capacity between 2-62 ng/mg for E1 applied at concentrations of 3.6-65 ng E1/l (Ong *et al.,* 2001). The adsorption of E1 is linear in a buffer solution, whereas using surface water and STP effluent this is not the case. The latter is due to a preloading with other organics adsorbing to PAC as well. In a buffer solution with a concentration of 100 ng E1/l, a concentration of 5 mg/l PAC was removing over 80% of E1, and at 20 mg/l more than 95%, whereas for surface water containing E1 100 ng/l 80% removal was only achieved at a PAC concentration of 50 mg/l which could not be achieved for STP effluent in this concentration. As a post-treatment system aimed for the removal of estrogens, the use of PAC may not be suitable, as a lot of PAC will be needed to achieve a sufficient removal. Also Fuerhacker *et al.* (2001) concluded that the adsorption of E2 to granular activated carbon (GAC) is insufficient as at equilibrium, only 49-81% of the E2 in the 1-100 ng/l range in deionised water is adsorbed.

2.7.2 Membrane filtration

Membrane filtration processes include microfiltration (MF, macropores >50 nm), ultrafiltration (UF, mesopores 2-50 nm), nanofiltration (NF, micropores <2 nm), reverse osmosis (RO, dense <2 nm), dialysis, and electrodialysis (ED) (Metcalf and Eddy, 2003). NF distinguishes itself from RO as it only retains multivalent ions, so it has an economic advantage when the retention of monovalent ions is not required (Schäfer *et al.,* 2003). The most important removal of estrogens by a membrane is by retention on the membrane or by adsorption to organic particulates which are then retained, since membrane pores are still larger than the radius of for example E1, which is 0.84 nm, whilst the average pore radius for a 1000 Da membrane is 0.94 nm (Schäfer *et al.,* 2002b). The adsorption capacity of the

membranes for hormones is affected by: type of membrane, pH, affinity of hormones to water, as well as the presence of other organic compounds (Chang *et al.,* 2002b).

A number of commercially available NF and RO membranes have been investigated for the retention of E1 dissolved in a carbonate buffer (Schäfer *et al.,* 2003). In general the retention at an initial concentration of 100 ng/l was very good, 95-99% with the exception of one, which was 80%. For the used membrane types, both size exclusion and adsorptive effects are responsible for maintaining high retention of E1 in this type of membrane. Adsorptive effects appear to be particularly important for retention by NF membranes exhibiting relatively low ion retentions. These adsorptive effects may be driven by hydrogen bonding between E1 and the membranes (Schäfer *et al.,* 2003). The adsorption on hydrophobic membranes is higher than on hydrophilic material. This was confirmed by Chang *et al.* (2002b) for E1. No difference between an ionic strength of 0.02 M and 0.2 M was found, and the pH was only affecting sorption above a pH-value of 11 (Chang *et al.,* 2002b). The affinity for the membrane decreases when the estrogens become charged at high pH, since they are both negatively charged. E1 removal in a buffer solution showed higher removal compared to E1 removal in surface water and secondary effluent, indicating a possible competition with other organic compounds, although the removal was not influenced dramatically. The retention on the membrane decreases with the increase of the concentration on the surface and a breakthrough will occur when the concentration on the surface reaches the equilibrium value for the corresponding feed concentration (Chang *et al.,* 2002a/b).

With MF or UF membranes, pore sizes are too big and the main removal mechanism will be adsorption to the membrane and particulates, which is low at neutral pH and decreased at pH higher than 10.5 (Schäfer and Waite 2002). Estrone retention was found higher in presence of suspended organic matter (Schäfer *et al.,* 2002b; Schäfer and Waite, 2002). Therefore the effects of adsorbents used during wastewater treatment; such as powdered activated carbon, ferric chloride coagulant and magnetic ion exchange resins (MIEX®) have been investigated (Schäfer and Waite 2002). This research confirmed again that the addition of ferric chloride does not change the E1 concentration. With a relatively low dose of activated carbon (5-10 mg/l) over 80% removal of E1 is achieved. MIEX can bind small organic pollutants and polar compounds. Adding MIEX the removal of E1 is up to 45% and increased with the pH. When the molecules are dissociated at a pH above 10.4 and negatively charged, the removal is up to 70 % (Schäfer *et al.,* 2002a; Schäfer and Waite 2002). Increasing the pH to 10.4 is no option for municipal wastewater, but might be applicable for urine treatment.

In an attempt to increase the adsorption of estrogens to membranes, antigens were build-in a porous hollow fibre membrane for the removal of E2, breakthrough was depending on the ligand density in the membrane, which was at most 5.8 mg/mg glucidyl methacrylate fibre (Nishiyama *et al.,* 2002). The molar binding ratio was very low (0.043) and the amount of E2 bound to the fibre decreased with an increasing number of cycles, which might indicate that the binding capacity is decreasing, possibly by the use of the eluent fluid (10% methanol/water). It is clear that insufficient data are available for any application of hollow fibre filtration in the current state. Research within the multidisciplinary DeSaR research next

to present research and performed at Utwente, showed very promising results for affinity membranes with build-in an E2-antigens (Urmenyi *et al.*, 2005).
Microfiltration had been compared to a microfiltration PAC hybrid system for the removal of E1 from a buffer solution (Ong *et al.,* 2001). Without the addition of PAC, the membrane was saturated after approximately one hour and shows a decrease in E1 retention, whilst with the addition of PAC the removal is a function of PAC concentration, higher concentration leads to a faster establishment of the equilibrium.

When using a membrane in combination with an activated sludge system, a so-called membrane bioreactor, enhancing effects on the removal of estrogens could be expected because of an increased biomass concentration and the longer SRT (Wintgens and Melin, 2001). At the time of writing the review, no information was available on pilot- or full scale membrane bioreactors. Currently a number of researches are known which all indicate an efficient removal of estrogens in MBR-systems, especially when NF and RO membranes are applied (Kim et al., 2007), but due to the shorter HRT, overall removal rates are not necessarily better compared to activated sludge systems (Hu *et al.,* 2007).Clara *et al.* (2005) did not find any difference in the performance of an MBR compared to a conventional STP regarding the removal of estrogens.

2.7.3 Ozonation and advanced oxidation processes

Ozonation and especially advanced oxidation processes (AOP) are used to convert complex organic compounds in wastewater to decrease their toxicity (Metcalf and Eddy 2003). AOP uses hydroxyl free radicals (HO·) as a nonselective oxidant. They are formed by using ozone and UV, or ozone in combination with hydrogen peroxide or hydrogen peroxide in combination with UV. In a few cases AOP is tested for the conversion of estrogens in final effluents as described below.

Even after tertiary treatment of municipal wastewater effluent, including sand filtration, ozone and UV, E2 and E1 were still detected in the effluent in a concentration of 0.8 ng/l and 1.3 ng/l respectively, whilst EE2 stays below the detection limit of 5 ng/l in both influent and effluent (Chapman, 2003). Thus, separate ozonation might not be as effective as AOP. There are no further specifications given about the treatment steps itself, so making it difficult to draw a conclusion from this research. Applying 10-15 mg/l ozone with a contact time of 18 minutes, it is able to remove E1 in a concentration of 15 ng/l to below the detection limit from STP effluent from an activated sludge plant treating municipal wastewater in Germany (Ternes *et al.,* 2003).

E2 is highly reactive towards ozone because of the two reactive hydroxyl groups (Kosaka *et al.,* 2000). During treatment with O_3/H_2O_2, ozone is more selective than HO·, and since E2 is a highly reactive target, it will be removed quite easily even in presence of radical scavenging compounds such as humic acid (Kosaka *et al.,* 2000). Also EE2 can be attacked by ozone and the half-life was 5 μs under typical ozonation conditions (Gunten *et al.,* 2003; Huber *et al.,* 2003).

In a study comparing sand filtration, ozone/hydrogen peroxide (AOP), micro-filtration and reverse osmosis for the removal of estrogenity from municipal wastewater, only AOP and

reverse osmosis were able to remove total estrogenic activity for over 97% whilst in the other options, the removal was insufficient (Shishida *et al.,* 2000).

2.8 Conclusions

From the three studied compounds, E1, E2 and EE2, the degradation rate of EE2 was considerably lower compared to E1 and E2. Sorption of EE2 to sludge was higher compared to E1 and E2. Degradation rates are higher in a temperature range from 20-25°C compared to 5-10°C. Sludge with a higher SRT shows a faster degradation of E1, E2 and EE2. The phenomenon of increased degradation rates at lower initial concentration should be investigated in more detail in order to find a suitable explanation.

The adsorption constants for different types of sludge would be useful to determine for the aid of mass balances for full-scale plants. The constants can be determined by separately measuring the estrogens adsorbed on sludge and dissolved in the liquid phase. To exclude biodegradation and photolysis it is recommended to perform the test with inactivated sludge in the dark. Inactivation should not lead to a change of the sludge structure, because it can have influence on the available binding places.

Although the risk for bioaccumulation of natural estrogens from domestic wastewater in the environment is expected to be small, this risk can be significantly higher for the synthetic hormone EE2, due to its slower degradation and higher tendency to sorb. In order to prevent estrogenic effects on fish, especially in cases where there is little effluent dilution, the improvement of STPs regarding the removal of estrogens will be a long-term solution.
Another issue that deserves attention is the way sludge is dealt with. During anaerobic sludge treatment the estrogens are hardly removed. In case sludge is used on (farm)land, estrogens might run-off with the rainwater and end up in streams.

Although activated sludge is able to biodegrade estrone, 17β-estradiol and 17α-ethynylestradiol, they are still present in final effluents in the ng/l range. In order to obtain more fundamental knowledge on removal processes, more attention should be paid to parameters that can influence the conversion rate of estrogens, like the (actual) HRT, SRT and loading rate. This knowledge can contribute to an optimisation of existing treatment plants, rather than addition of costly tertiary treatment steps. Another important research area is the development, standardization and validation of determination methods for estrogens in the wastewater and sludge matrix. As there is no standardised method to measure E1, E2 and EE2 in the wastewater and sludge, it would be advantageous to introduce such a method to ease the comparability of different researches. Also there seems to be a lack of research for “real” wastewater, especially in case of tertiary treatment.

Acknowledgements

The authors thank Simon Stocking for the assistance in the preparation of the manuscript.

Chapter 3

Analytical procedures for the determination of estrone, 17β-estradiol and 17α-ethynylestradiol in wastewater and sludge matrices

Abstract

Prior to analytical detection of estrogens in environmental samples, a number of procedural steps to diminish matrix effects and to increase the concentration, are required. Current research applied HPLC -analyses with UV, fluorescence and diode array detection for analyses of estrogens in a concentration range of 3 μg/l up to 20 mg/l, whereas environmental relevant concentrations were measured on a GC-MS in a range of 12 ng/l to 50 μg/l. The estrogen concentrations in the liquid and the solid (sludge) phase were analysed separately. Total concentrations (liquid+solid) were obtained by extraction of freeze-dried samples. Information on the amount of conjugated estrogens could be obtained by subjecting a sample to an enzymatic solution, which converted the conjugated estrogens back to their measurable unconjugated forms. The applied methods were shown to be satisfactory

3.1 Introduction

Determination of natural and synthetic estrogens in wastewater and sludge is difficult as samples contain very low concentrations of these compounds and the matrices are complex, therefore the execution of the determination requires a considerable number of procedural steps (López de Alda and Barcelo, 2001). The steps generally followed for the analytical determination of estrogens in wastewater and sludge are summarised in Figure 3.1. They can be separated in:

1. sample pre-treatment, i.e. the separation of suspended material from the liquid phase to enable determination of the target compounds in the liquid and the solid phase;
2. extraction of the target compounds from the solid phase;
3. enrichment of the target compounds from the liquid phase and the extracts prepared from the solid phase by solid phase extraction (SPE);
4. a clean up step to remove humic substances and/or colloidal particles that remained after SPE e.g. with a silica gel column;
5. reconstitution of the sample by re-dissolving the dried sample with a known amount of solvent and transfer into a sample vial;
6. detection and quantification.

In addition, when the detection method is not able to quantify estrogens in the conjugated form, an enzyme solution can be used to hydrolyse the conjugates to their unconjugated form.

For the determination of estrogens, glass is preferred over synthetic laboratory equipment, in order to prevent adsorption and leaching of interfering chemicals. In present research the used glassware was rinsed with HPLC grade methanol (Acros, the Netherlands) prior to use to remove interfering pollutants. Often glassware is subjected to an additional pre-treatment with a silane solution, which is filling up active binding sites on the glass wall, preventing adsorption of estrogens (Belfroid *et al.,* 1999a). After cleaning with methanol, in the present research glassware was rinsed with Millipore water, since pre-tests showed a better recovery of estrogens, when rinsing with Millipore water than compared to silane solution (Appendix I).

In the following paragraphs every step for the determination of the concentration of estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) is described in detail.

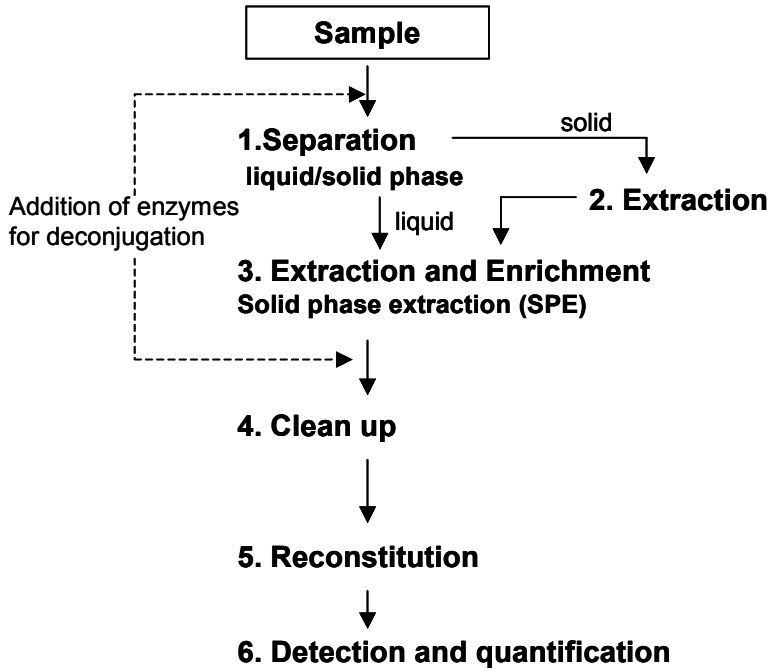


Figure 3.1. Schematic overview of the procedure for pre-treatment, pre-concentration and cleaning up for analyses of estrogens in wastewater and sludge samples.

3.2 Sample pre-treatment

3.2.1 Sample preservation

To prevent biological degradation of estrogens during storage, preservatives can be added to the sample. Frequently, acidification is applied by adjusting the samples pH to 2 or 3 (Isobe *et al,.* 2003; Rutishauser *et al.,* 2004) with H_2SO_4 or HCl (McArdell *et al.,* 2006). In an acidic environment the estrogens will be present in undissociated form, which might increase the affinity during SPE. On the other hand, Ferguson *et al.* (2001) did not find any difference in extraction efficiency by SPE with a C18 cartridge between acidified and non-acidified (neutral) samples in tests with an isotope of E2: [3H]-17β-estradiol. Since acidification can affect the stability of some analytes, some care should be taken (Reemtsma and Quintana, 2006).

In current research, all examined samples were strived to be processed the same day, since this is considered to be the best practice (McArdell *et al.*, 2006). Processed samples were stored in 1,5 ml crimp sealed vials at 5°C and analysed within a week. When samples could not be processed the same day, sodiumazide (NaN_3), an often used preservative (method 4500-O, (APHA/AWWA, 1998)), was added at a concentration of 0.5 g NaN_3/l (Merck Hohenbrunn) and the samples were stored at 5°C.

3.2.3 Filtration and centrifugation

Often, raw wastewater samples are filtered through a glass fibre filter prior to solid phase extraction (SPE) (Ternes *et al.,* 1999b; Johnson *et al.,* 2000; Laganà *et al.* 2000; Andersen *et al.,* 2003; D'Ascenzo *et al.,* 2003) to prevent clogging of the SPE disk or filter. This can induce an underestimation of the total amount of estrogens present in a sample as only the dissolved concentration is assessed.

In current research, generally no glass fibre filtration was applied for wastewater samples without a high fraction of colloidal or suspended solids. For samples with a high colloidal or suspended solids content, the samples were filtered using a Whatman GF/C glass fibre filter. The complete glass fibre filter was extracted and analysed for the presence of estrogens.

Separation of the solid and liquid phase in sludge samples was conducted by centrifugation; a sample (typically 40 ml) was centrifuged at 3000 relative centrifugal force (rcf) for 10 minutes (IEC, Centra, CL3 and Heraeus instruments, Labofuge 400). After centrifugation of the sample, 30 ml of the supernatant was taken out with a glass pipette and collected in a clean glass bottle, representing the liquid phase. The rest of the liquid phase was carefully removed with a Pasteur pipette. The solid and the liquid phase were always individually enriched by SPE and analysed.

The amount of liquid still present in activated sludge after centrifugation (40 ml, in quatro) was quantified by determination of the dry weight of the sludge pellet after 24 hours drying in a 105°C oven (WTC binder). The amount of liquid present in the pellet after the liquid has been removed, induced a small overestimation of the concentration in sludge. A sludge pellet, obtained from 40 ml sludge mixture, contained on average 1.80±0.04 ml of liquid. This liquid is also consisting of intracellular fluid, and the maximum amount of overestimation at an initial estrogen concentration of 5 mg/l is 4.8±0.1%, amounting to 0.24 mg/l.

3.2.3 Sludge extraction

The classical extraction procedure of solid samples, the Soxlet extraction, has been nowadays replaced by microwave assisted extraction (Liu *et al.,* 2004), supercritical fluid extraction, pressurized liquid extraction and ultrasound assisted extraction (Reemtsma and Quintana, 2006). The last mentioned method was applied in present research.

Set-up

For sludge extraction, the sludge pellet obtained after centrifugation of the sample, or a freeze dried sample (Chapter 3.2.4), was subjected to four extractions with a methanol:acetone (MeOH:Ace; 1:1) mixture. The first extraction consisted of addition of 10 ml MeOH:Ace, after which the slurry was sonificated (Transsonic T460/H from Elma) for 10 minutes. Next, the sample was centrifuged (3000 rcf, IEC Centra CL3) and the liquid layer is collected in a clean glass bottle. In next three extractions, 5 ml MeOH:Ace was used, and the sonification and centrifugation steps were repeated. In order to confirm that sufficient recovery of estrogens is achieved by four subsequent extractions, the following test has been performed: a 200 ml activated sludge sample from the full-scale STP of Bennekom was spiked with EE2 at a concentration of 4.93 mg/l. The sludge sample was diluted to a total suspended solid (TSS)-content of 0.83±0.19 g/l and a volatile suspended solid (VSS)-content of 0.59±0.16 g/l. The TSS- and VSS-contents were determined according standard method 2540D/2540E (APHA/AWWA, 1998). After one hour mixing on a magnetic stirrer, two samples of 40 ml (in duplo) were taken and the sludge was separated from the liquid by centrifugation as described in Chapter 3.2.2. In each centrifuged sample, EE2 was analysed in 30 ml of the supernatant. The sludge pellet was extracted five times according to above described method.

Millipore water was added to the individual extracts up to a total volume of 40 ml, in order to enable SPE as described in Chapter 3.4. SPE was applied directly to the 30 ml liquid samples. After SPE, the samples were dried completely under a gentle stream of nitrogen gas and reconstituted with 1.5 ml MeOH. Samples were analysed by HPLC with UV detection, according to the method described in Chapter 3.8.1.

Results

Figure 3.2 shows the yielded EE2 after each extraction step. Of the 1,46 mg/l EE2 attached to the sludge up to 92% is already extracted by the first extraction step, 7% by the second step, only 1% by the third step, while in the fourth and fifth extraction steps EE2 was not detected. The measured amount in the liquid phase amounted to 3.58±0.11 mg/l and the sum of the extracts gave 1.46±0.25 mg/l, so in total 5.04±0.27 mg/l, which resulted in a recovery of 102%.

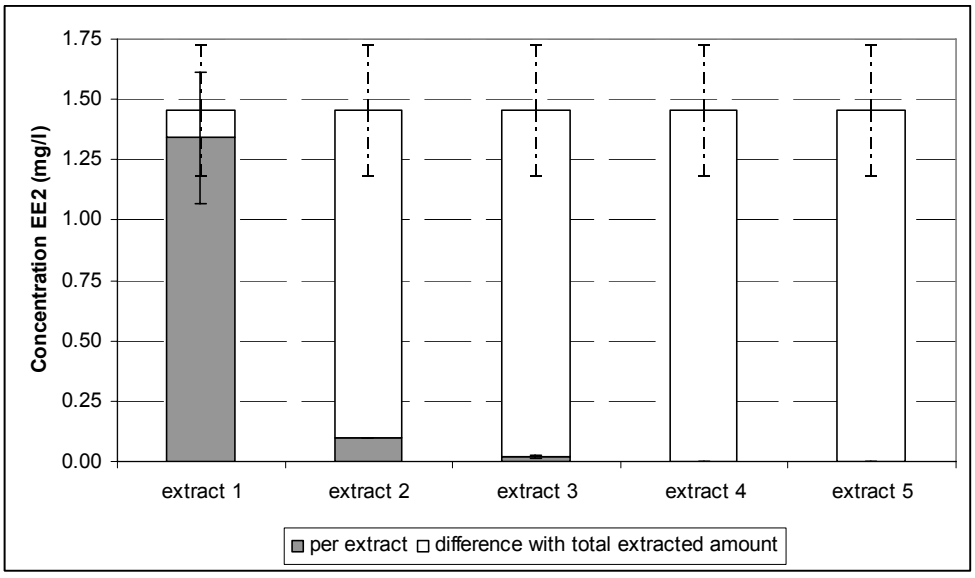


Figure 3.2. Determined EE2 concentration in each individual sludge extract (grey bars) to the total extracted amount (white bars) from an activated sludge sample (40 ml) that was spiked with EE2.

Based on this results it can be concluded that the applied sludge extraction technique indeed gave a complete recovery of estrogens from the sludge. Four extraction steps for sludge extraction are sufficient to extract all estrogens from the sludge phase at applied estrogen concentrations.

3.2.4 Freeze drying

To obtain the total amount of estrogens in a sludge or wastewater sample, freeze drying can be used (Ternes *et al.,* 2002). In current research, before the sample was put in a freeze dryer, a known amount was collected in aluminium bowls of 1-L and covered with paper as lid. The sample was pre-frozen in a freezer at -20°C. After freeze drying (GRI freeze-dryer, GR Instruments, Driebergen, the Netherlands; temperature program ranging from -15°C to +10°C under vacuum) a known amount of dried sample was extracted according to the procedure for sludge extraction described and evaluated in Chapter 3.2.3. The time needed for freeze drying

depended on the volume and amount of samples put in the freeze dryer. Regular visual checks were done to observe whether the samples were indeed completely dry.

3.3 Enzymatic hydrolysis of conjugated estrogens

Conjugated estrogens can be measured by LCMS, whereas GC-MS only enables detection of unconjugated estrogens. For the analyses of conjugated estrogens by GC-MS, a hydrolysis step with an enzyme solution is needed for deconjugation of all the estrogens. The concentrations of conjugated estrogens could then be determined from the difference obtained between the results for hydrolysed and unhydrolysed samples. The enzyme solution can be added either directly to the sample or to the extract after SPE.

SPE is expected to have a low affinity for conjugated estrogens, because of their increased water solubility, compared to unconjugated estrogens. Legler *et al.* (2002) demonstrated in experiments with pure urine that enzyme addition to acetonitrile SPE extracts resulted in a two to three times lower concentration of total estrogens compared to the case where enzymes were applied to pure urine. Therefore it is recommended to add the enzymatic mixture to the sample prior to SPE.

Only a few researches so far paid attention to the presence of conjugated estrogens in domestic wastewater. In present research the deconjugation method was based on Legler *et al.* (2002) and Huang and Sedlak (2001). The enzyme, H-1 from Sigma by *Helix Promatia*, the Netherlands, exhibits both β-glucuronidase and sulphatase activity, viz. respectivively ≥300,000 units/g and ≥10,000 units/g (information provided by the manufacturer). Typically, between 1 and 20 units of glucuronidase is used per µl of urine (*Helix promatia*, the Netherlands). Legler *et al.* (2002) used 40 units per ml urine and Huang and Sedlak (2001) applied between 1.6 and 11 units per ml municipal wastewater treatment effluent where the majority of estrogens is likely to be present in unconjugated form.

In present research, 20 units of enzyme were applied per ml concentrated black water, collected with vacuum toilets using one litre water per flush. The enzymatic solution was prepared in a 0.1 M of acetate/acetic acid buffer at pH 5.0. The buffer contained 6.23 g sodium acetate and 1.44 ml glacial acetic acid, in which the pH was adjusted to 5.0 with 0.1 M NaOH. The stock solution contained 1000 units/ml and 1 ml was enough to add sufficient enzymatic units for a 50 ml sample of black water.

As the pH-optimum of the reaction is 5.0, an extra 20 ml of acetate/acetic acid buffer was added to the sample prior to addition of 1 ml enzymatic buffer. The sample was placed on a magnetic stirrer for 20 hours and the temperature was controlled at 37°C.

3.4 Enrichment by Solid Phase Extraction

Solid phase extraction (SPE) is a necessary step for enrichment of the target compounds and to diminish matrix effects (Reemtsma *et al.,* 2006). Extraction cartridges are generally applied for SPE, but clogging rapidly occurs when used for wastewater samples, mainly due to the presence of colloidal and particulate matter (Kelly, 2000). Cartridges often contained additional contaminants, leached from the plastic walls of the cartridges themselves (Kelly, 2000). To overcome these problems in present research, SPE disks were used.

When using HPLC with UV-VIS, DAD and FLU as the detection method, C18 SPE disks (3M Empore, diameter of 47 mm) were used. When GC-MS(-MS) was used as a detection method, SDB-XC (styrene-divinylbenzene) disks (3M Empore, diameter of 47 mm) were used, as they are yielding a better enrichment compared to C18 disks (personal communication Ms. P. Booy, IVM, Amsterdam).

For conditioning the disks and elution of the estrogens from the disks, manufacturers' instructions were followed. After placing the C18 disk on a filtration unit (equipped with a vacuum pump) the disk was first rinsed two times with 5 ml acetonitrile, and then conditioned two times with 5 ml methanol for 30 seconds, leaving a thin layer of methanol on the disk. Next the filter is washed three times with 10 ml Millipore water after which a thin layer of liquid remained on the disk. The sample is quantitatively filtered through the disk and, when dry, rinsed with 5 ml Millipore water. The filtrate is discarded and an empty glass bottle is placed under the disk, and subsequently the disk is eluted four times with 5 ml acetonitrile. The acetonitrile, which contains the estrogens then was completely evaporated. The dried sample was reconstituted with a known amount of solvent as described in Chapter 3.7. Conditioning and elution of SDB-XC disks proceeded in the same fashion as for the C18 disks, except that the disk was humidified with acetone prior to use and isopropanol was used instead of acetonitrile for the first rinsing.

Set-up

The performance of the C18-disks have been tested by spiking E1, E2 and EE2 (62, 52 and 59 mg/l respectively) to an extract of freeze dried UASB septic tank sludge before and after SPE. The sludge originated from a pilot scale UASB septic tank treating concentrated black water at 25°C. The extract was obtained from two different amounts of freeze dried UASB septic tank sludge (0.10 and 0.20 g), with 10 ml methanol/acetone (1:1), sonificated for 10 minutes. The supernatant was collected after 10 minutes centrifugation at 3000 rcf.

Results

The recovery was slightly better when less freeze dried sludge was used and only slightly increased when estrogens were spiked after SPE. From the results presented in Table 3.1 it can be concluded that at used concentrations, a maximum of 16% loss for E2+EE2 occurred during SPE extraction. Taking the standard errors of the used equipment (pipettes, balances) into account, a 6% of standard error can be calculated.

In addition to analytical determinations of samples, Millipore water containing all three estrogens were processed according to the same procedure as the samples and acted as a blank to account for any losses during the sample pre-treatment steps.

Table 3.1. Recovery of estrogens spiked before and after SPE in an extract of freeze-dried UASB-septic tank sludge.

	weight of freeze dried sludge (g)	% recovery E2+EE2	% recovery E1
Spiked before SPE	0.1036	94	92
	0.1991	84	84
Spiked after SPE	0.1036	105	99
	0.1997	90	89

3.5 Additional clean-up steps

After SPE the remaining organic material that could interfere in the detection method, can be removed with extra clean-up steps, like fractioning silica gel columns (Beck *et al.,* 2005), HPLC fractionation (Huang and Sedlak, 2001), additional SPE steps like C18 and fractioning in amino (NH_2) columns (Belfroid *et al.,* 1999b) and gel permeation chromatography (Ternes *et al.,* 2002).

In present research, silica gel columns have been used as additional clean-up step, according to Ternes *et al.* (1999b). To glass cartridges, with glass wool at the bottom, a slurry containing 1 g of silica gel in 4 ml hexane, was added (silica gel for column chromatography, 0.06-0.2 mm, pore diameter ca. 4 nm, Janssen Chimica, Belgium, deactivated with 1,5% Millipore water). The silica was left over night at 550°C prior to use. The silica gel column was then rinsed with 10 ml petroleum ether and pre-concentrated extracts evaporated till approximately 200 µl after SPE were quantitatively transferred to the column. The estrogens were eluted using 6 ml petroleum ether after which the samples were evaporated to dryness. For the subsequent reconstitution see Chapter 3.7.

3.6 Derivatisation

Derivatisation makes the compounds more volatile, which enables better detection with a GC-MS. Shareef *et al.* (2004, 2006) demonstrated that the agents as well as the solvents used in the procedures, are of major importance and can cause incomplete derivatisation as well as the occurrence of multiple peaks, also EE2 derivates can be converted into E1 derivatives. Zuo and Zhang (2005) demonstrated a complete and pure derivate was formed when proper ratio's sample and derivatisation agent were used. Gómez *et al.* (2006) applied no derivatisation prior to GC-MS-MS measurements, but applied high injection volumes of 8 µl, whereas in general only 1 µl was used.

In present research, the completely evaporated samples after SPE and/or additional clean-up, were derivatised for 1 hour at 60°C with 25 µL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MFSTFA; Sigma), containing 4 mg 1,4-dithioerythritrol (Merck) and 2 mg ammonium iodide per ml. After derivatisation the fluid was evaporated under a gentle stream of N_2-gas and reconstituted in HPLC-grade petroleum ether (Suprasolv, Merck). The derivatisation agent was prepared in two steps (A and B), where the actual applied derivatisation agent B, was prepared from agent A. Derivatisation agent A was prepared, containing 40 mg 1,4-dithioerythritrol (Merck) and 20 mg ammonium iodide per ml MSTFA which has a shelf life of 1 month at 5-10°C. Derivatisation agent B was prepared with 100 µl of derivatisation agent A en 900 µl MFSTA and was freshly prepared prior to use (personal communication Mr. B. Brouwer, Rikilt, the Netherlands).

3.7 Reconstitution of the sample

After SPE enrichment, the completely evaporated sample was transferred to a sample vial by re-dissolving with a known amount of solvent. For HPLC-analyses, in general 1.5 ml of methanol was used. For GC-MS analyses the nearly dried sample (to about 100 µl after SPE)

is quantitatively transferred to a 1.5 ml conical vial and then completely evaporated before being subjected to derivatisation. As methanol was found in present research to destabilise derivates, petroleum ether was used as a solvent. A higher concentration factor was reached by adding less solvent for reconstitution. When samples are used in the YES bioassay, reconstitution is done with the non toxic and non-volatile dimethylsulfoxide (DMSO) (Murk *et al.*, 2002).

3.8 Detection and quantification methods

Chromatography (Tandem) Mass spectrometry (GC-MS(-MS)) is an often used technique for the detection and quantification of estrogens in surface water, wastewater or sludge, after the volatility of the compounds has been increased by a necessary derivatisation step. Also Liquid chromatography combined with mass spectrometry (LC-MS-MS) nowadays is an often used method for determination of the steroid estrogens (Zuehlke *et al.,* 2005; Richardson, 2006). The main advantage of this method is that derivatisation is not required, whilst still very low limits of detection can be achieved (around 1 ng/l) (Cui *et al.,* 2006). Moreover, it is possible to detect conjugated estrogens without applying a deconjugation step. New developments can be expected with High Performance Liquid Chromatography (HPLC)-time of flight (TOF) mass spectrometry. A TOF analyser can provide a more accurate mass estimation, which allows better distinction between estrogens and matrix (Reddy and Brownawell, 2005). Another way of quantification is HPLC followed by a UV-(ultra violet visible) detector (UV-VIS), Diode Array Detector (DAD) or Fluorescence (FLU) detector. The reconstituted sample can also be exposed to a bioassay, e.g. a Yeast Estrogen Response (YES), in which the total estrogenity of a sample is expressed as E2-equivalents (Witters *et al.*, 2001; Murk *et al.,* 2002). Although the YES method was demonstrated to be very suitable for the identification of real-life effects in environmental samples, viz. surface waters, soils, sediments, wastewater (Murk *et al.,* 2002; Onda *et al.,* 2002; Saito *et al.,* 2002; Tilton *et al.,* 2002), it was found to be inappropriate in this research to elucidate fate during wastewater treatment, like adsorption and biodegradation (Cordoba, 2004). This can be attributed to a high background noise in the samples as well as toxicity of wastewater and sludge to the yeast.

At the start of the research only HPLC with UV-detection was available and due to the high detection limit and limited sensitivity and accuracy, the sorption and biodegradability tests were performed at relatively high spiking concentrations of 5 mg estrogens/l. In a later phase of the research, a fluorescence detector was placed in series with the UV detector, which improved the sensitivity and consequently lowered the limits of detection. Towards the end of the research the UV detector was replaced by a diode array detector (DAD), improving the selectivity by obtaining a spectrum of a compound. In order to enable measurements of estrogens in unspiked samples, it was necessary to develop a highly sensitive and selective method by using a GC-MS. Also GC-MS-MS measurement was tried, but - despite the expectations - did not attain further improvement of the sensitivity in the investigated matrix of anaerobically treated concentrated black water.

3.8.1 HPLC with UV-VIS, Fluorescence and DAD detection

Estrogens were determined in an HPLC set-up consisting of a Marathon auto sampler, a Gynotek high precision pump, model 480 Elite™ with an Alltech degassing system used to lead the mobile phase (0.4 ml/min) over a 1 cm C18 pre-column with a diameter of 6 mm, and next over two 10 cm Chromopack columns with outer diameter of 6 mm and inner diameter of 5 mm. The absorbance was measured using a programmable UV absorbance detector (Kratos analytical, type spectroflow 783), at 200 nm. A programmable fluorescence detector (Hewlett Packard HP1046A), was placed in series with the UV detector. The extinction of the fluorescence detector was 230 nm and the emission 310 nm and a cut-off filter was filtering out compounds with a spectrum below 305 nm. The injection volume was 10 µl and the mobile phase consisted of 60% acetonitrile and 40% Millipore water (Millipore Simplicity 185, consisting of a Simpack®1) when solely EE2 needed to be detected. For the detection of E1 and/or E2 or all three compounds, 40% acetonitrile and 60% Millipore water was used as mobile phase.

3.8.2 GC-MS(-MS)

GC-MS(-MS) analyses were performed on an Interscience system (Breda, the Netherlands) consisting of a Trace GC 2000 gas chromatograph equipped with a PTV (programmable temperature vaporization) split-splitless injector, and AS 2000 sampler, and a Polaris Q ion-trap mass spectrometer (Thermofinnigan, Breda, the Netherlands). The injector was equipped with a 120 mm x 2 mm I.D. SilcoSteel liner (Interscience). Injection volumes of 2 µl of a derivatised extract or standard solution were used. The PTV valve was operated in the splitless mode. Compounds were separated on a 50 m x 0.25 mm I.D. low bleed-MS column coated with a 0.25 µm film of CDP-Sil 8 CB (95% dimethyl-5% phenyl polysiloxane; Varian Chrompack, the Netherlands). Helium was used as carrier gas at a constant flow of 1 ml/min. The injector, ion source and mass transfer line temperatures were 250°C, 250°C and 300°C respectively. Best separation and peak shape were derived by the following temperature programme viz. initial temperature 30°C (boiling point of petroleum ether), first ramp with 30°C/minute to 130°C, second ramp with 2°C/minute to 268°C, on hold for 10 minutes, then the last ramp with 20°C/minute to 300°C and on hold for 5 minutes to clean out the column (Noppe, 2006).

Table 3.2. shows the molar masses of (derivatised) E1, E2 and EE2 used for GC-MS quantification. The retention times of the target compounds can vary between different runs and are always reconfirmed by the calibration standards. Figure 3.3 provides an example of a chromatogram in a full scan mode. Figures 3.4, 3.5 and 3.6 show the mass spectrums for E1, E2 and EE2 respectively in which the relative abundance of the masses used for quantification (Table 3.2) can be clearly distinguished.

Table 3.2. Molecular masses used for GC-MS analysis of estrone, 17β-estradiol and 17α-ethynylestradiol.

Name compound	Retention time (minutes)	Molecular mass	Molecular mass derivates	*m/z*[a] used for quantification
Estrone (E1)	26.81	270.4	414	218, 257, 342, 414
17β-estradiol (E2)	27.41	288.4	416	129, 285, 326, 401, 416
17α-ethynylestradiol (EE2)	31.04	296.4	440	285, 300, 425, 440

[a] m/z mass to charge ratio; the mass number of an ion divided by its charge number.

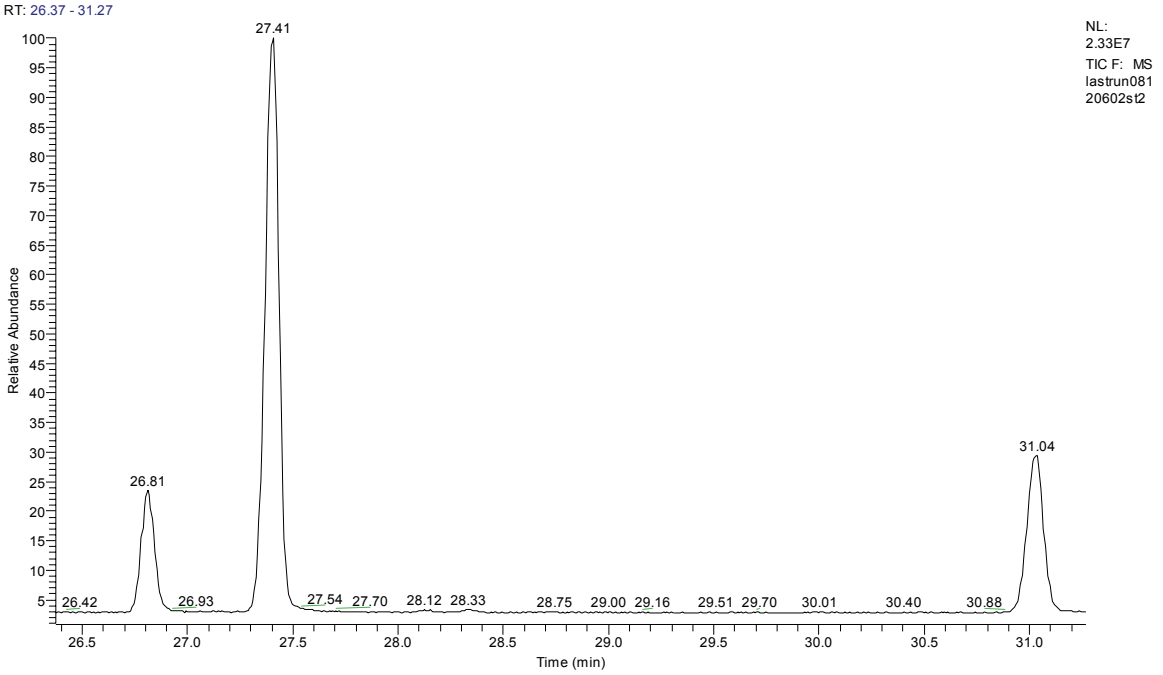


Figure 3.3. Example of a GC-MS chromatogram after derivatisation; retention times (RT): E1, E2 and EE2 are 26.81, 27.41 and 31.04 minutes, respectively.

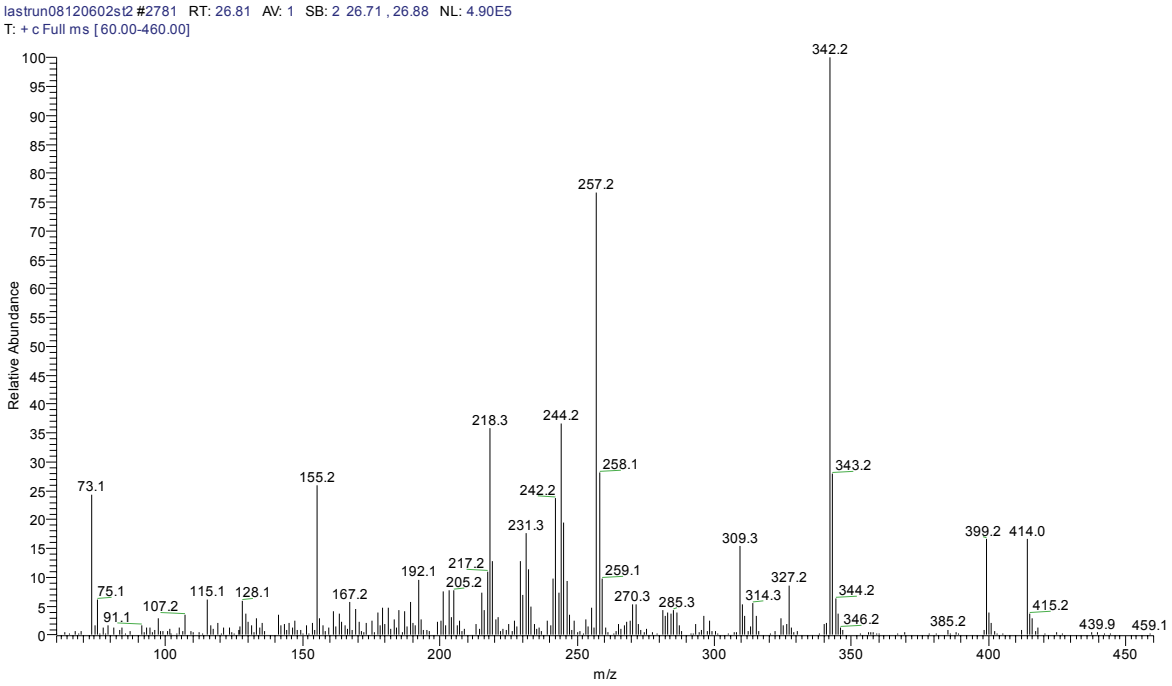


Figure 3.4. Mass-spectrum of derivatised E1 (the relative abundance for the m/z distribution at RT 26.81 min).

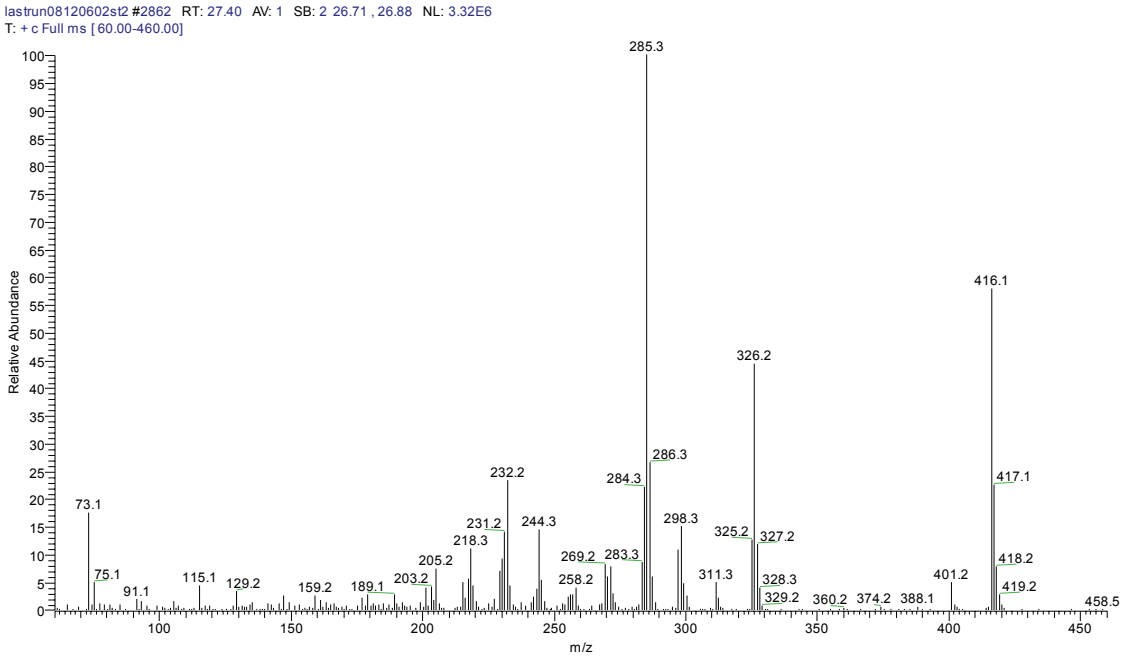


Figure 3.5. Mass-spectrum of derivatised E2 (the relative abundance for the m/z distribution at RT 27.41 min).

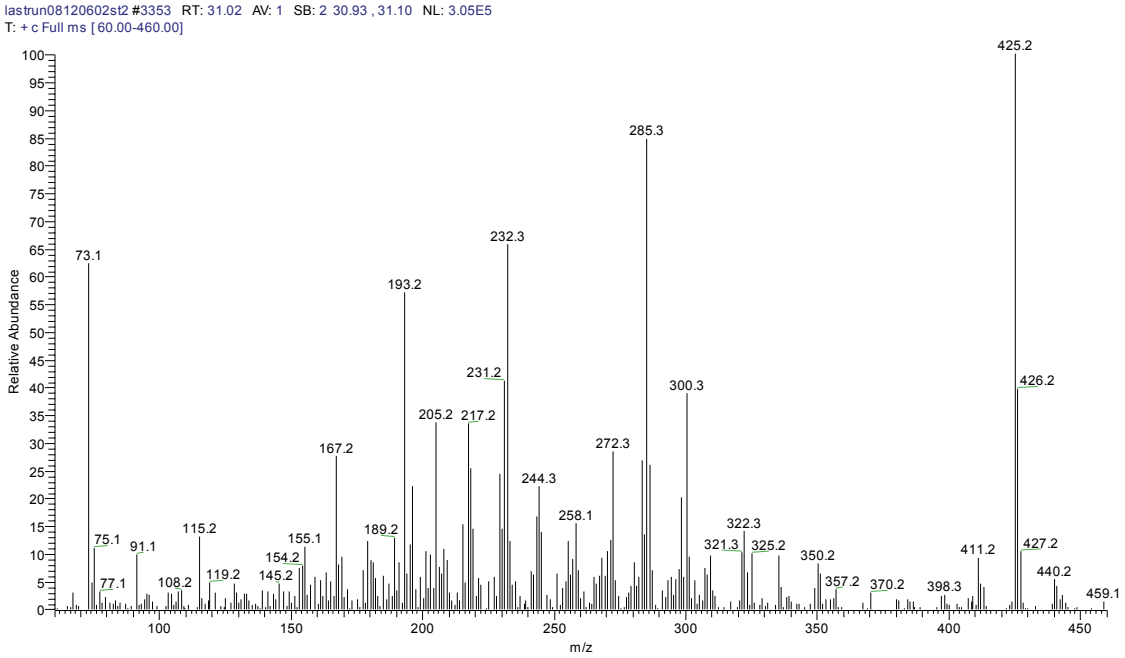


Figure 3.6. Mass-spectrum of derivatised EE2 (the relative abundance for the m/z distribution at RT 31.04 min).

Figure 3.7 shows the total scan chromatogram of an anaerobically treated concentrated black water spiked with E1, E2 and EE2. This figure reveals that, when no filter is used for the specific masses for quantification (Table 3.2), the EE2 peak can not be distinguished, due to other components in this matrix with the same retention time. The peaks of E1 and E2 are also more profound when the chromatogram is only showing their respective masses from Table 3.2. Figures 3.8, 3.9 and 3.10 are showing very clear peaks for estrogens enabling their quantification.

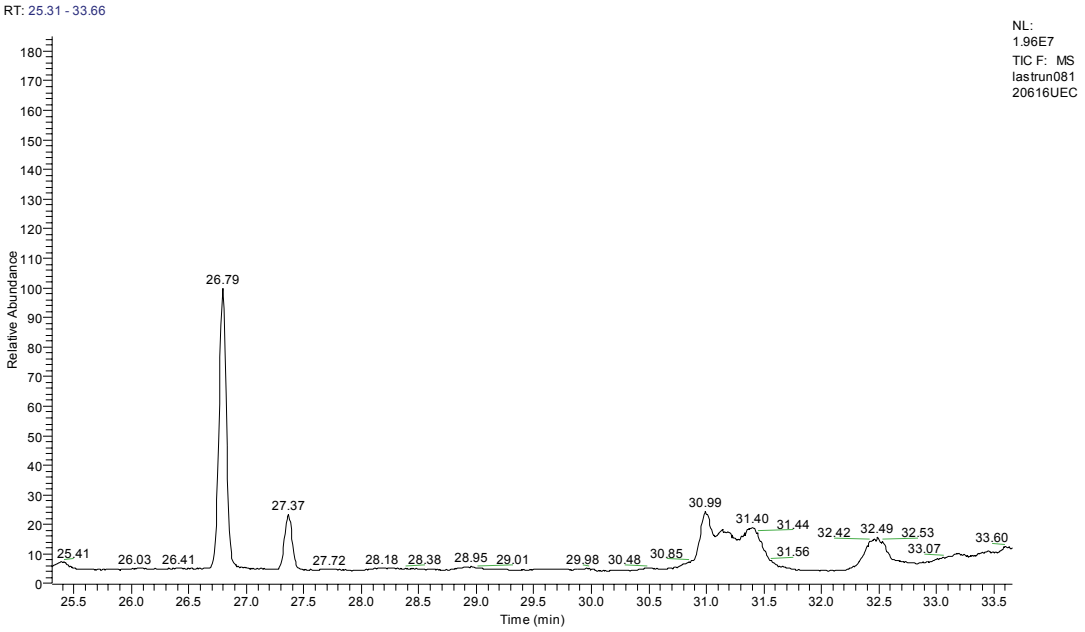


Figure 3.7. Spiked sample UASB septic tank effluent, chromatogram of the whole mass range (m/z).

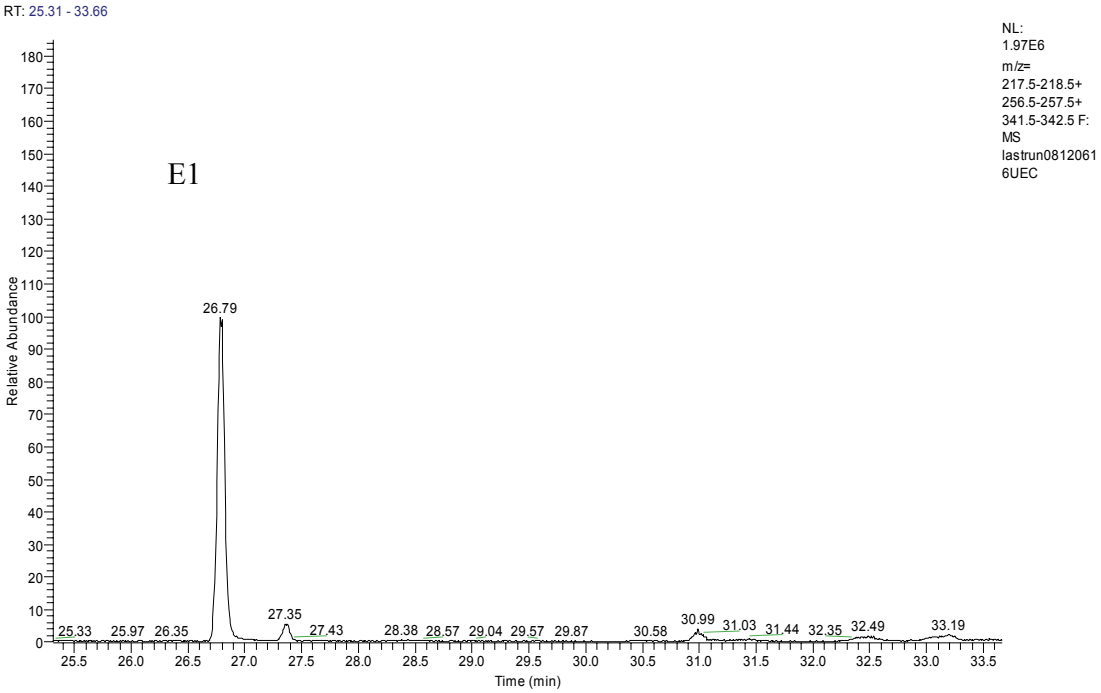


Figure 3.8. Spiked sample UASB septic tank effluent, chromatogram of the masses for E1 quantification (*m/z* 218, 257, 342, 414).

3.8.3 Sample quantification and quality control

The limit of detection (LOD) is the smallest amount, or concentration of the analyte in a test sample that can be reliably distinguished from zero (Thompson *et al.,* 2002). LODs are higher for STP effluents than for surface waters as the matrix of the STP effluents is more complex (Belfroid *et al.,* 1999b). The limit of quantification (LOQ) usually is three times the LOD value, or as reported by Jeannot *et al.* (2002) the signal-to-noise ratio, which compares the level of a desired signal to the level of background noise, is S/N=10. Other ways for determination of the LOQ are based on the lowest calibration point of the calibration curve

after accounting for the sample pre-concentration or standard deviation of a number of injections multiplied by ten (Jeannot *et al.,* 2002). The LOD for the GC-MS method was determined by multiplying the noise level of the baseline with the factor 3. LOD and LOQ values of the used methods are summarized in Table 3.3 and the concentration ranges that were applied for each method are depicted in Figure 3.11.

A calibration curve was prepared with end concentrations in the vial of 500, 100, 50, 20 and 2.5 µg/l of each individual estrogen, which underwent derivatisation. Calibration curves approached linearity (R^2>0.95), but concentrations were calculated with the non-linear option available in the XCalibur software.

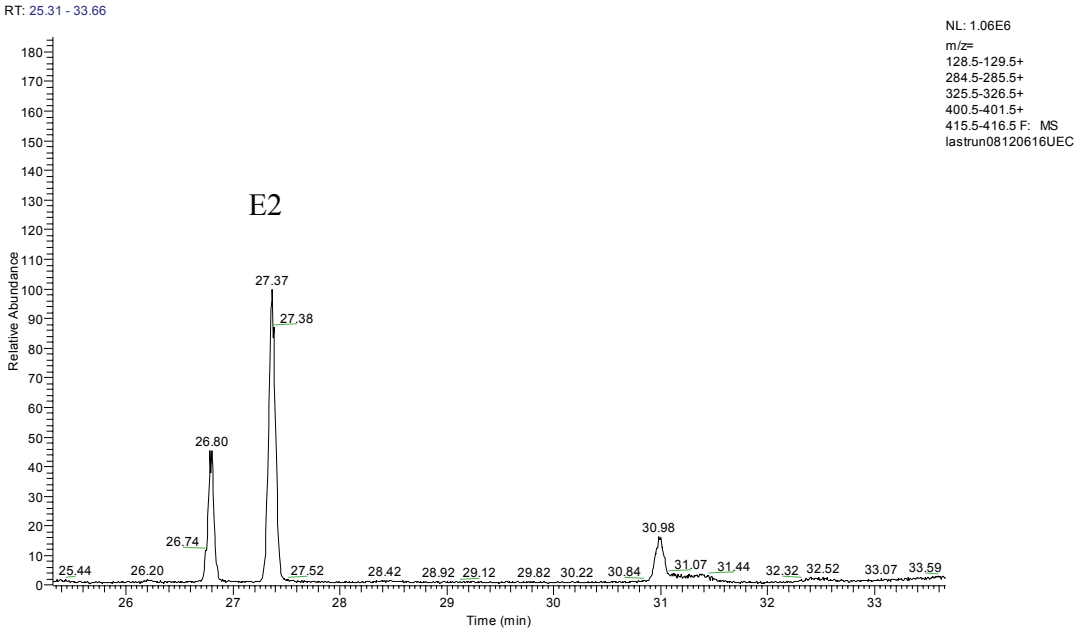


Figure 3.9. Spiked sample UASB septic tank effluent, chromatogram of the masses for E2 quantification (*m/z* 129, 285, 326, 401, 416).

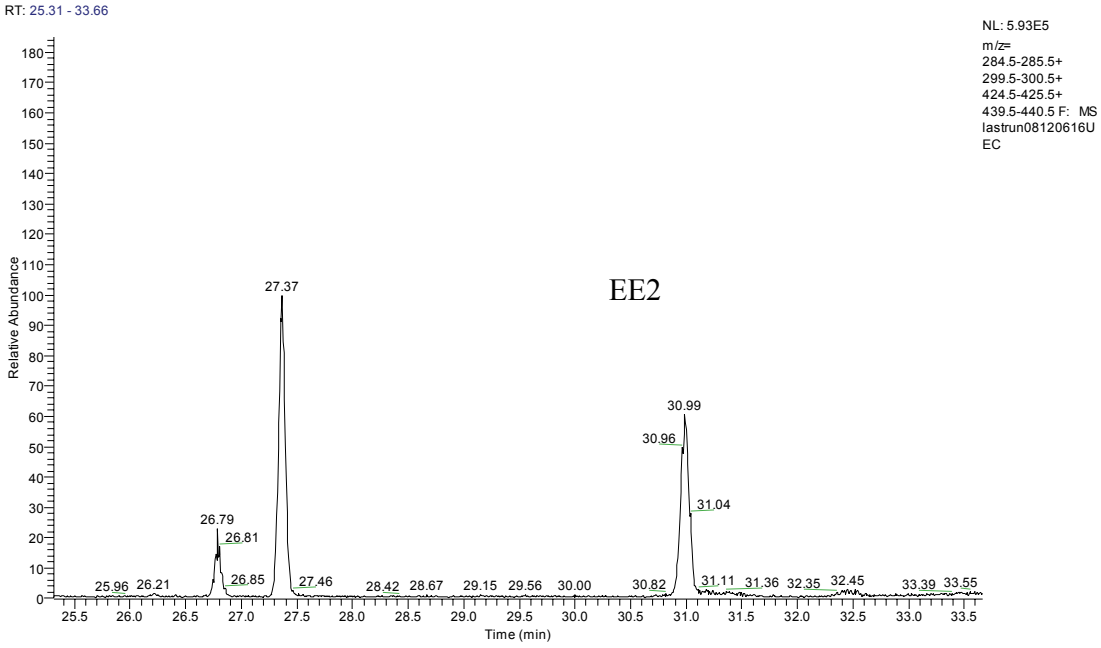


Figure 3.10. Spiked sample UASB septic tank effluent, chromatogram of the masses for EE2 quantification (*m/z* 285, 300, 425, 440).

One of the most important but least recognized sources of error in analytical measurements can be attributed to matrix variations (Thompson *et al.,* 2002). These can have consequences in the sample pre-concentration because e.g. binding places on the SPE disk during solid phase extraction can be occupied by other compounds present in the matrix, or positive or negative matrix effects during detection, which can result in an over- or under-estimation of the compound. The best way to deal with these obscure matrix effects is to work with isotope labelled internal standards. Present research used Mirex (Dr. Eherenstorfer GmbH, Germany) as internal standard (IS), especially during the development of the method, resulting in good recoveries. As the compound is chemically completely different as the targeted estrogens, the suitability of this IS is doubtful.

In current research the matrix effect in the GC-MS method was accounted for by splitting the reconstituted sample into two and spiking one with a known amount of derivatised standard. Besides that, in all cases the spiked blanks were analysed parallel with analysis of the samples in order to account for losses during all the applied steps and to assess the recovery factor. When the recoveries were low, a repeat was performed, when possible.

Table 3.3. Determined limits of detection (LODs) and limits of quantification (LOQs) in used detection methods for different matrices.

	matrix	E1		E2		EE2	
		LOD	LOQ	LOD	LOQ	LOD	LOQ
UV-VIS 200 nm	liquid	10	30	15	45	25	75
DAD-UV-VIS 200 nm		n.d.[a]	n.d.	n.d.	n.d.	5	15
DAD-UV-VIS 210 nm		n.d.	n.d.	n.d.	n.d.	20	60
FLU		20	60	8	24	1	3
UV-VIS 200 nm	sludge	10	30	80	240	40	120
DAD-UV-VIS 200 nm		n.d.	n.d.	n.d.	n.d.	6	18
DAD-UV-VIS 210 nm		n.d.	n.d.	n.d.	n.d.	7	21
FLU		5	15	10	30	2	6
DAD-UV-VIS 200 nm	Tenax[b]	0.5	1.5	0.1	0.3	1	3
DAD-UV-VIS 210 nm		1	3	3	9	6	18
FLU		0.5	1.5	0.3	0.9	0.7	2.1
GC-MS	All	0.02	0.05	0.05	0.15	0.10	0.30

[a] n.d.= not determined [b] industrial sorbent used for determination of bioavailability and desoprtion (Cornelissen *et al.,* 1997 and Chapter 5 of this thesis)

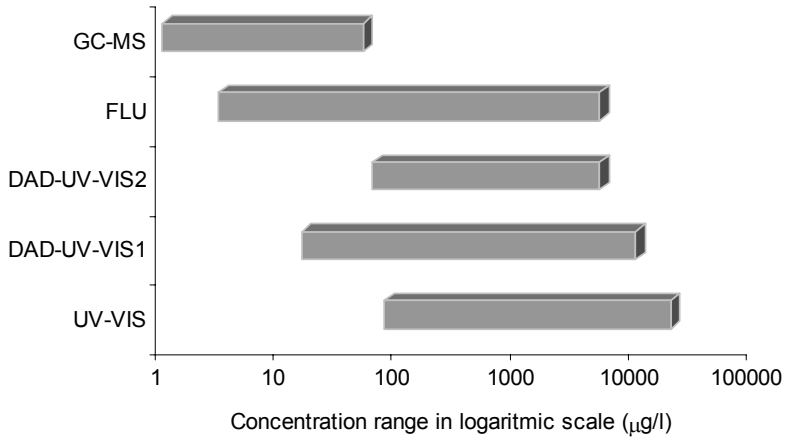


Figure 3.11. Concentration ranges as applied for the different detection methods.

3.9 Concluding remarks

The development of a reliable methodology for measurement of estrogens in wastewater, and more specifically in concentrated black water, appeared to be an extremely time consuming and complex task. Since the start of current research in 2001, several methods to determine estrogens at low concentrations in environmental matrices has been developed. All developed methods consist of a number of analytical procedural steps prior to actual detection. This is not only time consuming, but it also introduces risks for errors. This necessitates an extensive control protocol (blanks, matrix effect correction, standards). In general, it can be stated that the simplest procedures and mechanisms have a privilege, as simpler techniques allow to conduct a greater number of analyses (Umbreit, 1983). Especially as variability in the sample itself is expected, it is more desirable to have more results with a moderate accuracy rather than fewer results with a higher accuracy (Umbreit, 1983).

Although current research could not entirely keep up with the latest developments, the applied methods are shown to be satisfactory. As estrogens are moderately hydrophobic, and therefore adsorption to organic material will occur, separate analyses were always performed on both the solid and the liquid phase. The applied method for sludge extraction was demonstrated to be very suitable. According to the latest development where a microwave assisted method (Liu *et al.,* 2004) is used, with recoveries of 61% on average for the extraction of estrogens from river sediments, whilst the method in current research for sludge obtained recoveries of >85%. Solid phase extraction for the enrichment of the estrogens was very satisfactory for both C18 as SBD-XC disks, i.e. on average a recovery of >90% was obtained. Reconstitution of the samples is a very critical step because it can induce a very large error. When concentrating the sample to a high extent, e.g. a 1-L sample down to a 100 µl concentrate, the test result has to be multiplied by 10.000 to obtain the original concentration. Thus when the 100 µl end volume differs 10 µl, already a 10% error is induced.

As detection method, LCMS and TOF offer the advantage over GC-MS that no extra derivatisation step is needed. Moreover, these methods are also capable to detect conjugated estrogens. However, care has to be taken during the analytical procedural steps, as conjugates might not be stable and convert back to their original unconjugated form. In current research GC-MS detection was shown to be a very suitable method for the determination of estrogens in a concentrated black water matrix. But it would be very attractive to develop methods with a lower number of procedural steps and the ability to detect conjugated estrogens.

Chapter 4

Fate of estrone in presence of aerobic and anaerobic sludges under various redox conditions

Submitted for publication (de Mes *et al.*, in preparation)

Abstract

Estrone (E1) was selected for research as higher concentrations for E1 compared with 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) were measured in influent and effluent of wastewater treatment plants. Degradation rates for E1 were assessed with activated sludge in batch experiments under aerobic, micro-aerobic, inhibited nitrifying, anoxic and anaerobic conditions. The overall first order degradation rates (*k*-values) were calculated for each condition. Additionally, a distinction was made between a rapid and a slow degradable fraction, with their accompanying degradation rates, k_{rapid} and k_{slow}. Aerobic conditions were most favourable for E1 degradation with a calculated mean specific *k*-value of 0.27 l.g $TSS^{-1}.d^{-1}$. Under micro-aerobic conditions the degradation rate was significantly lower with a specific *k*-value of 0.10 l.g $TSS^{-1}.d^{-1}$. Present experiments were performed at relatively high initial E1 concentrations (mg/l range). Degradation rates were significantly lower as compared to those calculated from experiments in which low initial E1 concentrations (ng/l and μg/l range) were applied. The rapidly degrading fraction comprised only 0.15 under aerobic conditions, with an accompanying specific-k_{rapid} of 101 l.g $TSS^{-1}.d^{-1}$. A rapid transformation of E2 into E1 under aerobic conditions occurred with a specific *k*-value of 97 l. g $TSS^{-1}.d^{-1}$. Anoxic conditions resulted in a low E1 degradation rate of approximately 0.004 l.g $TSS^{-1}.d^{-1}$. Under anaerobic conditions the dominant process was the reduction of E1 into E2 without a decline of the sum of E1 and E2. Even in long-term experiments (205 d) under anaerobic conditions in presence of either digested pig manure, granular UASB sludge, UASB septic tank sludge or activated sludge, no decline in the sum of E1 and E2 was observed. The rate and extent of reduction of E1 to E2 did vary between the different sludges. Degradation of E1 was not significantly slower under inhibited nitrifying conditions compared to aerobic conditions with nitrification. The calculated adsorption coefficients (K_f values) for the adsorption of E1 to sludge indicate that sorption plays an important role in the removal of E1 from the liquid phase.

4.1 Introduction

Present research focused on the fate of estrone (E1), as higher concentrations of this specific compound were found in influents and effluents of sewage treatment plants (STPs), when compared to 17β-estradiol (E2) and 17α-ethynylestradiol (EE2). Values for E1 also show highest variability between different STPs and within the same STP over time. Measurements in full scale STPs as presented in Chapter 2 give average effluent concentrations of 13.9±14.3 ng/l for E1, 2.1±2.7 ng/l for E2 and 1.7±4.3 ng/l for EE2. The average removal percentages are 62±27% for E1, 88±9% for E2 and 56±24% for EE2. These figures were calculated using data from research of Lee and Peart (1998); Baronti *et al.* (2000); Adler (2001); Bruchet *et al.* (2002); Schullerer *et al.* (2002); Vethaak *et al.* (2002); Andersen *et al.* (2003); Cargouët *et al.* (2004). One reason for the higher concentrations of E1 compared to E2 is the higher excretion rate, viz. 13.8 μg.$person^{-1}.d^{-1}$ for E1 and 3.3 μg.$person^{-1}.d^{-1}$ for E2 (Johnson and Williams, 2004). Estrogens are mainly excreted in urine (Williams and Stancel, 1996) as sulphate (21%) or glucuronide conjugates (79%) (D'Ascenzo *et al.*, 2003) and 5-10% unconjugated (parent compound) in faeces (Adlercreutz and Järvenpää, 1982). Conjugates are biologically inactive (Ingerslev and Halling-Sørensen, 2003), but bacterial enzymes can hydrolyse them back to their original form (Ternes *et al.*, 1999a). Unlike the deconjugation of glucuronide conjugates, which already occurs in the sewer, the deconjugation of sulphate conjugates mainly occurs

during treatment in the activated sludge process (D'Ascenzo *et al.*, 2003). In final effluents 60% of the compound is present in unconjugated form (Adler, 2001; D'Ascenzo *et al.*, 2003). As current analytical techniques mainly determine the unconjugated active compounds, influent concentrations of STPs are underestimated. Additionally, rapid transformation of E2 into E1 under aerobic conditions attributes to the relatively high E1 effluent concentrations (Ternes *et al.* 1999a; Baronti *et al.*, 2000). E1 will ultimately become mineralized as was shown by Layton *et al.* (2000) and Jürgens *et al.* (2002) in experiments with ^{14}C labelled estrogens.

This research investigated the fate of E1 in activated sludge under various redox conditions, viz. aerobic, micro-aerobic, anoxic and anaerobic. Moreover the effect of nitrification inhibition was investigated, as according to Vader *et al.* (2000) nitrifying bacteria may play a crucial role in the conversion of these hormones. The degradation of E2 was merely investigated under aerobic conditions in order to confirm its rapid transformation to E1. Besides, the digestibility of E1 was investigated under anaerobic conditions in presence of four different sludges.

Based on the moderately high octanol-water partition coefficient (K_{ow}=3.4 ;Lai *et al.*, (2000)), adsorption is expected to play an important role in the removal of E1 from the liquid phase in treatment systems. Adsorption parameters for E1 were obtained by a short-term adsorption experiment in activated sludge. Knowledge on the effect of exposure of E1 to anaerobic conditions is of importance with regard to the growing interest in the implementation of new sanitation concepts, where source separated concentrated streams are subjected to anaerobic (pre-)treatment processes (Zeeman and Lettinga, 2001). Moreover, in conventional treatment approaches anaerobic digestion is frequently applied for the stabilization of primary and secondary sludge. In developing countries anaerobic treatment systems are increasingly applied for pre-treatment of raw domestic wastewater (Haandel and Lettinga, 1994; Foresti *et al.*, 2006).

4.2 Materials and Methods

Biodegradation of E1 is, like the degradation of other micro-pollutants (Temmink, 2001), presumed to follow first order reaction kinetics shown as Equation 7 in Chapter 2. From the *k*-value the half-life ($t_{1/2}$, d) can be calculated according to Equation 8 from Chapter 2 and the specific degradation rate, according to Equation 1.

$$\text{specific } k = \frac{k}{TSS_0} \qquad \text{Equation 1}$$

In which:

specific *k:* (l.g $TSS^{-1}d^{-1}$)

TSS_0: total suspended solids at the beginning of the experiment (g/l)

Secondly, the data were approached using Equation 2, which distinguishes between rapidly and slowly degrading fractions, with their accompanying degradation rates.

$$\frac{C_t}{C_0} = F_{rapid} \cdot e^{-k_{rapid} \cdot t} + F_{slow} \cdot e^{-k_{slow} \cdot t} \qquad \text{Equation 2}$$

In which:

C_t : concentration at time t (mg/l)
C_0 : initial concentration (mg/l)
F_{rapid} : fraction that is degraded at a rapid rate (-)
F_{slow} : fraction that is degraded at a slow rate (-)
t : time (d)
k_{rapid} : rapid degradation rate (d^{-1})
k_{slow} : slow degradation rate (d^{-1})

The adsorption process can be modelled with Equation 3 from Chapter 2, representing the Freundlich isotherm.The first order degradation rates, sorption coefficients, constants and the 95% confidence interval were calculated using non-linear regression in the statistical computer program SPSS 12.01. Determination of the adsorption parameters was conducted during the aerobic batch degradation experiments, and in separate short-term adsorption experiments (Andersen *et al.*, 2005).

4.2.1 Experimental set-up

Biological degradation

Degradation experiments were performed with sludge, spiked with a stock solution of E1 or E2 (CAS 63-16-7 and 50-28-2; Sigma-Aldrich, in HPLC grade methanol; Acros, the Netherlands, 5 g/l) to a concentration of 5 mg/l in 1-L serum bottles. The bottles were covered with aluminium foil to prevent photolytic degradation (Jürgens *et al.*, 2002). Five separate runs were conducted referred to as run A, B, C, D and F. The experimental set-up applied in this research to examine degradation constants were similar to Nyholm *et al.* (1996), who stated that "The first order degradation constants obtained from short term batch experiments with sludge without added sewage, are considered direct approximate estimates of biodegradation rates in activated sludge STPs".

Run A served for assessment of the degradation rates and adsorption of E1 under aerobic conditions. Samples were taken at 0, 4, 26, 118, 195 and 433 hrs. Run B was a replication of run A, but extended with assays conducted under anaerobic, anoxic, micro-aerobic and inhibited nitrifying conditions. Samples were taken at 0, 19, 122, 339 and 497 hrs. Run C was performed under aerobic and inhibited nitrifying conditions, while assays were conducted for assessing the influence of a two and four fold sludge dilution on the degradation kinetics. Samples were taken here at time 0, 2, 5, 22, 29, 48 and 70 hrs. In run D, the aerobic degradation of E2 was investigated in triplicate; sampling times were 0, 0.1, 0.7, 1, 2, 4, 6, 27 and 75 hrs. Run F comprised a long term experiment with anaerobic sludges of different origin, incubated at 30°C. Sample taking was done at t 0, 30, 45 days and in two sludge types even after 205 days. Oxygen-free conditions in the anoxic and anaerobic batches were maintained by flushing the capped bottles with nitrogen at the start of the experiment and after sampling. Anoxic conditions were created in the assays by supplying $NaNO_3$ (Merck, the Netherlands) at a rate of 14.4 mg N.g $VSS^{-1}.d^{-1}$. For inhibition of the nitrification,

20 mg/l N-Allylthiourea (ATU, Merck Hohenbrunn) was added every two days (Ning *et al.*, 2000). The pH, redox-potential, DO concentration and temperature were monitored at the sampling-times with portable meters (WTW). The TSS and VSS concentrations were measured at the beginning and at termination of the experiment, according to NEN 6621/6622 corresponding with standard method 2540D/2540E (APHA/AWWA, 1998). In the anaerobic long term experiment TS and VS concentrations were measured according standard method 2540B/2540E (APHA/AWWA, 1998).

Adsorption

A short-term adsorption experiment, run E, was performed in duplicate at four different E1 concentrations; 2.5, 5, 7.5 and 10 mg/l; samples were taken after one hour stirring at 25 ºC. According to Lai *et al.* (2000), after one hour the maximum sorption of estrogens to sediment will be attained. To prevent structural changes of the sludge, no sludge deactivation was applied.

Sludge origin and abiotic control

Activated sludge was collected from the low-loaded full scale nitrifying and biological phosphate removing STP of Bennekom, the Netherlands, operated at an sludge retention time of approximately 30 days. The sludge was sieved (2-mm pores) and incubated under aerobic conditions overnight prior to use. The sludge samples used for the long-term anaerobic experiment were: a) digested pig manure from a full scale manure digester located in Sterksel, the Netherlands, b) granular sludge from a full scale UASB treating wastewater from the paper industry, Eerbeek, the Netherlands, c) flocculent sludge from a pilot-scale UASB-septic tank fed with black water (Chapter 6; Kujawa-Roeleveld *et al.*, 2005) and d) activated sludge from the full scale oxidation ditch of Bennekom, the Netherlands.

Abiotic control was performed for E1 (5.0 mg/l) with autoclaved activated sludge (30 minutes at 121°C) after 17d and with demineralised water containing 0.5 g/l sodiumazide (NaN_3, Merck Hohenbrunn) after 10 d.

4.2.2 Chemical analyses

Well-mixed samples (40 ml) were analysed for E1 and E2 in both liquid and solid phase after separation, extraction, clean-up and pre-concentration by HPLC, in which E1 and E2 were separated over a 1 cm C18 pre-column (d=6mm) followed by 2 times 10 cm Chromopack columns (d=5 mm). The absorbance was measured by programmable absorbance detector from kratos analytical, type spectroflow 783, at 200 nm. The limit of detection (LOD) is 30 µg/l in a sample, when a pre-concentration using C18 solid-phase extraction disks (Varian, the Netherlands) of 27 times for sludge and 20 times for the liquid phase were applied.
In the long-term anaerobic experiment a different procedure was applied: The samples (20 g) were freeze-dried and extracted similar to solid phase samples. A detailed description of the method is reported by De Mes *et al.* (2006) and in Chapter 3 of this thesis. To confirm the occurrence of denitrification in the anoxic batch and inhibition of nitrification in the aerobic batch with ATU, ammonium, nitrate and nitrite were measured in paper-filtered samples

(Schleicher & Schuell, 5951/2 folded filters) at the beginning and end of the experiment, using a Skalar auto-analyzer according to ISO 11732 and ISO 13395.

4.3 Results and Discussion

4.3.1 Biodegradation of estrone and 17β-estradiol

Environmental conditions and abiotic control

Table 4.1 summarizes the prevailing environmental conditions in the experiments and the assessed concentrations of VSS and TSS. The average temperature was 23.3±2.5 °C for all experiments. Next to the DO concentrations, the redox values confirmed the targeted oxygen conditions. The calculated denitrification rate in the anoxic experiment was 11.1 mg $N.l^{-1}.d^{-1}$, based on the course of the measured ammonium, nitrite and nitrate concentrations (Appendix II); the value is in the typical range for sludge under endogenous conditions (Kujawa, 2000). Upon addition of ATU, nitrification was completely inhibited; no nitrite and nitrate were produced. The VSS concentration decreased as a result of the long-term endogenous conditions.

Table 4.1. Averaged values for DO and pH (with standard deviations), the range of the redox potential, VSS concentration at t_0 and at t_{end} and TSS concentration at t_0 in the different degradation and adsorption experiments, n.a.=not analysed.

Activated sludge, short-term		Duration experiment (hrs)	DO (mg/l)	Redox potential (mV)	pH	VSS_0 (g/l)	VSS_{end} (g/l)	TSS_0 (g/l)
Spiked compound E1								
A	Aerobic	433	7.7±0.5	61 to 174	6.9±1.3	2.30	1.47	2.95
B	Aerobic	498	9.0±0.5	151 to 224	6.7±1.2	2.60	1.78	3.34
C	Aerobic	70	8.5±0.4	125 to 142	7.8±0.4	2.90	2.02	3.68
C	Aerobic 2xdil.	70	9.2±0.1	113 to 202	8.0±0.4	1.45	0.94	1.84
C	Aerobic 4xdil.	70	9.3±0.1	126 to 163	8.1±0.5	0.72	0.41	0.92
B	Aerobic+ATU	498	8.8±0.6	20 to 71	7.7±0.2	2.60	1.66	3.34
C	Aerobic+ATU	70	8.7±0.5	90 to 127	7.9±0.5	2.90	2.26	3.68
B	Micro- aerobic	498	1.3±2.1	-36 to 131	6.5±0.7	2.60	1.87	3.34
B	Anaerobic	498	n.a.	-243 to -425	6.2±0.2	2.60	2.06	3.34
B	Anoxic	498	n.a.	-87 to -382	7.7±0.6	2.60	1.82	3.34
E	Adsorption	1	n.a.	n.a.	n.a.	n.a.	1.51±0.2[a]	1.93±0.3[a]
Anaerobic, long- term		Duration experiment (d)	Ammonium (mg N/l)	Redox potential (mV)	pH	VS_0 (g/l)	VS_{end} (g/l)	TS_0 (g/l)
F	Digested pig manure	205	4104	-443 to -188	8.6±0.8	10.5	8.9	21.5
F	Granular UASB sludge	45	121	-311 to -261	6.8±0.1	7.6	9.64	11.6
F	UASB septic tank sludge	45	709	-355 to -83	7.4±0.2	16.3	8.1	9.0
F	Activated sludge	205	68	-418 to -338	7.0±0.4	1.5	1.6	3.4
Spiked compound E2								
Activated sludge , short-term (heading similar as short-term experiments with E1)								
D[b]	Aerobic	75 (hrs)	2.2±1.0	41 to 263	7.2±0.2	2.31	1.86	3.11

[a]VSS and TSS determined at the end of the adsorption experiment (1h) [b]Values corresponding with explanation in the first row

In an autoclaved abiotic control experiment, the average loss of E1 amounted to only 6%, viz. the initial concentration 5.52±0.42 mg/l dropped to 5.18±0.15 mg/l at t_{end}. In the abiotic control conducted with demineralised water in presence of NaN_3, the E1 concentration remained almost unchanged, viz. the concentration at t_0 was 4.91±0.49 mg/l and t_{end} 5.01±0.36 mg/l.

Degradation of E1 and E2 under aerobic conditions

The course of the relative E1 concentration in time during the aerobic biodegradation experiment is shown in Figure 4.1A. The degradation rate for E1 under aerobic conditions is similar in all three runs (A, B and C), with a mean specific *k*-value of 0.27±0.04 l.g $TSS^{-1}.d^{-1}$, and a half-life of approximately 1 day (Table 4.2). The data in Table 4.2 and Figure 4.2 show no significant difference in degradation rates at higher E1/TSS ratios. The results in Table 4.2 and Figure 4.1B show that the aerobic transformation of E2 to E1 proceeds 300-times faster than the degradation of E1.

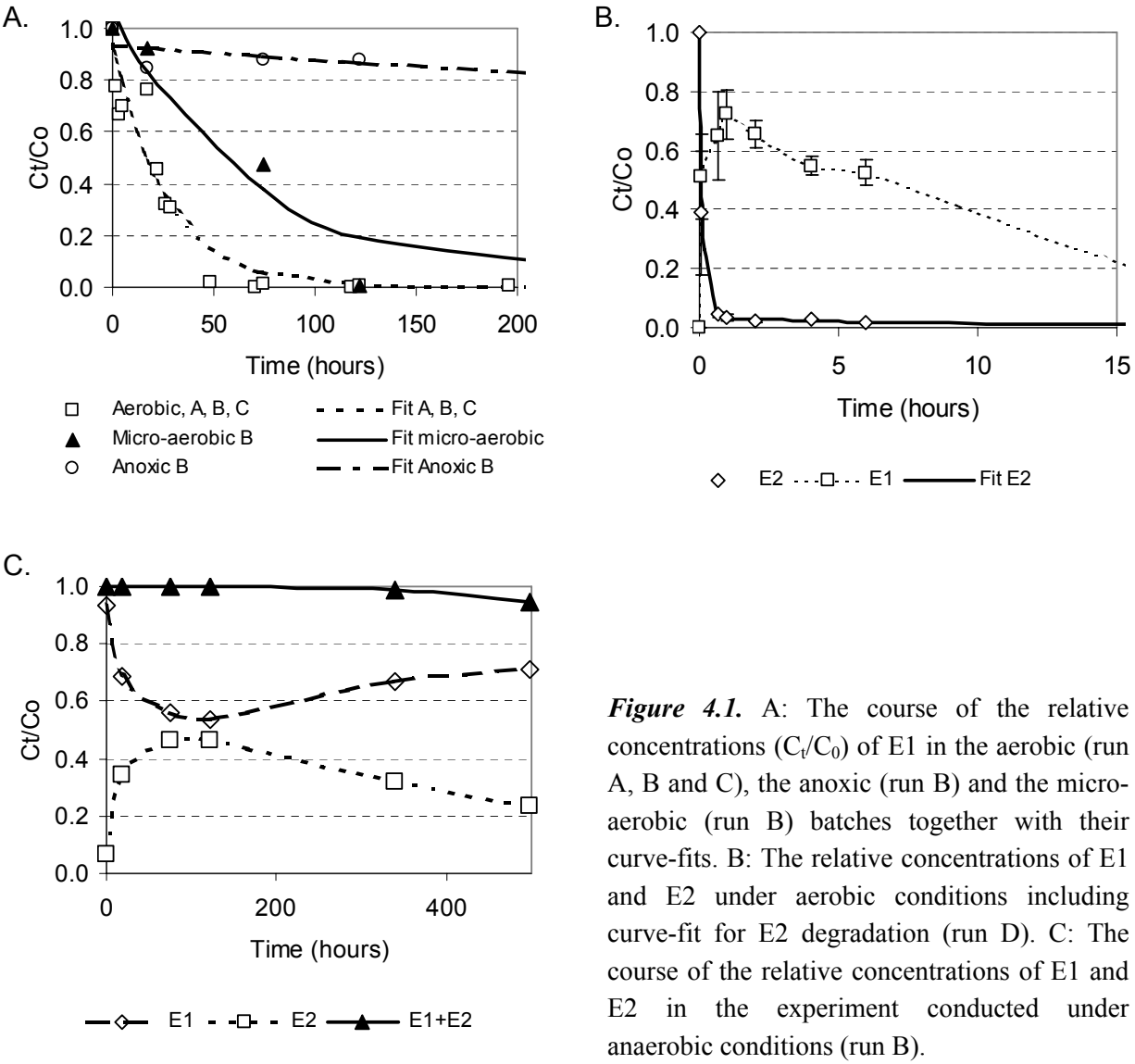


Figure 4.1. A: The course of the relative concentrations (C_t/C_0) of E1 in the aerobic (run A, B and C), the anoxic (run B) and the micro-aerobic (run B) batches together with their curve-fits. B: The relative concentrations of E1 and E2 under aerobic conditions including curve-fit for E2 degradation (run D). C: The course of the relative concentrations of E1 and E2 in the experiment conducted under anaerobic conditions (run B).

Under micro-aerobic conditions the calculated conversion rates are significant lower compared to those found under aerobic conditions (Figure 4.2).

Unlike the hypothesis of Vader *et al.* (2000), the results presented in Figure 4.2 reveal that the role of nitrifying organisms in activated sludge in the degradation of E1 is not significant. This is supported by Shi *et al.* (2004a), who reported a specific *k*-value of 1.13 l.g $TSS^{-1}.d^{-1}$ in presence of ATU and of 1.20 l.g $TSS^{-1}.d^{-1}$ in absence of ATU.

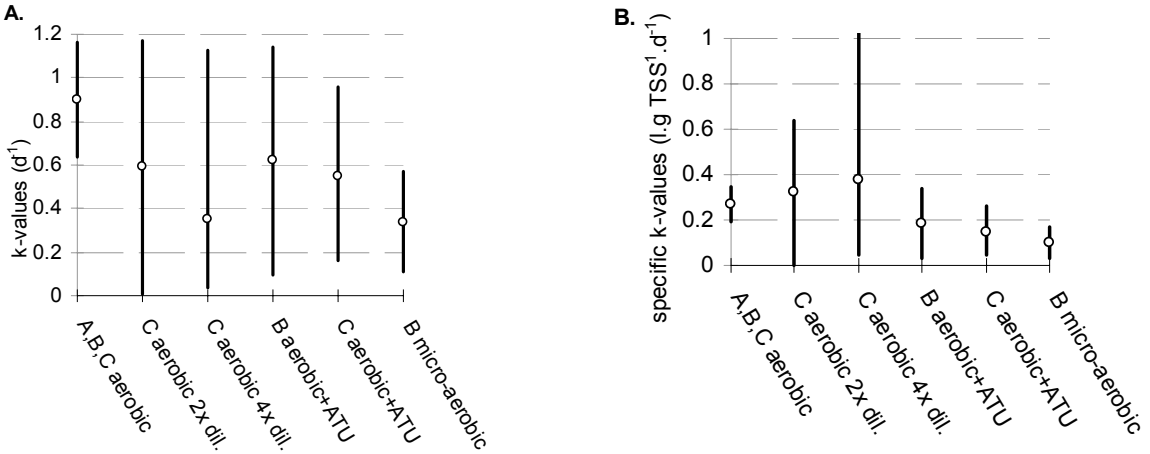


Figure 4.2 A: The 95% confidence interval of calculated first order degradation rates, *k*-values, for E1 degradation for imposed conditions. B: The 95% confidence interval of calculated specific first order degradations rates, specific *k*-values, for E1 degradation for imposed conditions.

Table 4.2. Calculated first order degradation parameters (including standard errors) and results of the two-stage approach for the fractions F_{rapid} and F_{slow} with accompanying degradation rates, k_{rapid} and k_{slow}, under different redox conditions at ambient temperature.

Run	Conditions	*k*-value (d^{-1})	$t_{1/2}$ (d)	Specific *k*-value (l.g $TSS^{-1}.d^{-1}$)	F_{rapid}	F_{slow}	k_{rapid} (d^{-1})	k_{slow} (d^{-1})
Degradation of E1								
A	Aerobic	1.10±0.26	0.63	0.37±0.09	0.25	0.75	317.3	0.79
B		0.67±0.15	1.03	0.20±0.04	Improper fit			
C		1.02±0.18	0.68	0.28±0.05	0.15	0.85	317.3	0.93
A,B,C	Average	0.90±0.14	0.77	0.27±0.04[b]	0.15	0.85	333.8	0.79
C[c]		0.59±0.21	1.17	0.32±0.11	0.27	0.73	298.6	0.50
C[d]		0.35±0.18	1.98	0.38±0.20	0.56	0.44	5.38	0.23
B	Aerobic+ ATU	0.62±0.26	1.12	0.19±0.05	Improper fit			
C		0.55±0.15	1.26	0.15±0.04	0.26	0.74	317.3	0.34
B	Micro-aerobic	0.34±0.08	2.04	0.10±0.02	Improper fit			
B	Anoxic	0.014±0.004	49.5	0.0042±0.0012	0.11	0.89	30.96	0.011
B	Anaerobic	0.0031±0.001	223.6	0.0009±0.003	Improper fit			
	E1 →E2	0.11±0.04	6.3	0.033±0.012	0.36	0.64	16.8	0.023
Degradation of E2								
D	Aerobic	303.6±46.1	0.002	97.2±14.8	0.98	0.02	319.4	2.88

[a] calculated without data point t1, as this was a suspected outlying value, [b] based on the average TSS experiments A, B and C, [c] two-fold diluted sludge, [d]four-fold diluted sludge

Effect of E1 concentration on the aerobic degradation rate

The *k*-values and specific *k*-values reported in literature for the degradation of E1 with activated sludge under aerobic conditions do not differ significantly when performed at similar initial E1 and TSS concentrations (Table 4.3). However, considerably higher conversion rates were found in experiments conducted at lower initial concentrations of E1 (μg/l and ng/l range). Ternes *et al.* (1999a) and Shi *et al.* (2004a) observed higher degradation rates at lower initial concentration of E1 (Table 4.3). This can be due to either incomplete extraction of E1 from sludge, which in fact implies that part of the presumed degradation may result from adsorption, or due to limiting desorption rates at high spiking concentrations. The relation in Figure 4.3 between the initial E1 concentration and the specific *k*-values clearly show highest conversion rates at lowest initial E1 concentrations. The occurrence of a rapid and slow degradation of E1 indicate that a substantial fraction of E1 is not directly available for degradation. The calculated k_{rapid} value in this research of 334 d^{-1} corresponds to a *k*-specific of 101 l.g $TSS^{-1}.d^{-1}$, and is similar to *k*-specific values reported for the degradation of E1 at low initial concentrations (Table 4.2 and 4.3). According to Nyholm *et al.* (1996) the observation of higher degradation rates at lower initial concentrations can be explained by the role of transient sorption, which for aniline became significant at concentrations below 20 μg/l.

Table 4.3. Calculated *k*-values and half-lives ($t_{1/2}$) at different initial E1 concentrations for the degradation of E1 in activated sludge under aerobic conditions at ambient temperature.

Initial E1 concentration	Ratio E1/TSS	*k*-value	Specific *k* – value	$t_{1/2}$	Reference
High (mg/l)	(mg/g)	(d^{-1})	(l.g $TSS^{-1}.d^{-1}$)	(d/ g TSS)	
20-25	10-12.5	0.31	0.16	4.5	Shi *et al.* (2004b)
20-25[a]	10-12.5	0.20	0.10	7.0	Shi *et al.* (2004b)
5.0	3.4	1.10	0.37	1.9	this research
5.0	2.8	0.67	0.20	3.5	this research
5.0	2.5	1.02	0.28	2.5	this research
5.0	5.3	0.59	0.32	2.2	this research
5.0	12.2	0.35	0.38	1.8	this research
1.0	0.4	1.34	0.50	1.4	Shi *et al.* (2004a)
0.2	0.3	0.86	1.20	0.6	Shi *et al.* (2004a)
1.0	3.8	1.01	3.88	0.2	Ternes *et al.* (1999a)
0.1	0.04	2.62-6.31	0.98-2.28	0.3-0.7	Urase and Kikuta (2005)
Medium (μg/l)	(μg/g)		(l.g $TSS^{-1}.d^{-1}$)	(min)	
1	1.9	11.52	22	45	Ternes *et al.* (1999a)
16[b]	8	100.56	50	20	Onda *et al.* (2003)
Low (ng/l)	(μg/g)		(l.g $TSS^{-1}.d^{-1}$)	(min)	
500[c]	1	202.56	405	2.5	Kjølholt *et al.* (2004)
500	1	48.60	162	6.2	Joss *et al.* (2004)
500[d]	1.7	124.70	430	2.3	Joss *et al.* (2004)

[a]night-soil composting sludge, [b]E1 only measured in the supernatant, [c]fed with artificial wastewater, [d]membrane bioreactor sludge

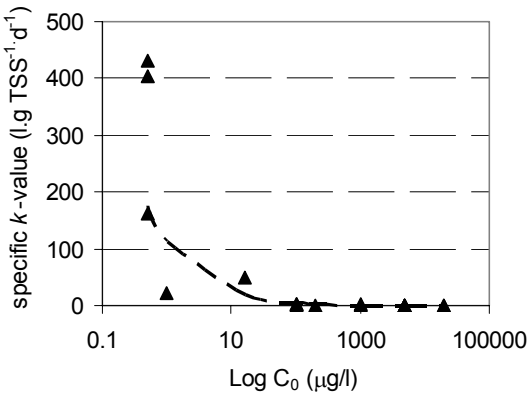


Figure 4.3. The relation between the initial E1 concentration (C_0) and the calculated specific *k*-value (l.g $TSS^{-1}.d^{-1}$) for aerobic degradation of E1 (data from Table 4.2 and 4.3).

Degradation of E1 under anoxic conditions

The results in Figure 4.1A show little degradation of E1 under anoxic conditions. The calculated specific *k*-value was 0.0042±0.0012 l.g $TSS^{-1}.d^{-1}$ (Table 4.2), which is distinctly lower than under aerobic, micro-aerobic and inhibited nitrifying (aerobic+ATU) conditions. Kjølholt *et al.* (2004) reported a specific *k*-value of 14±5 l.g $TSS^{-1}.d^{-1}$ ($t_{1/2}$=1.2 h) and Joss *et al.* (2004) 30±10 l.g $TSS^{-1}.d^{-1}$ ($t_{1/2}$=0.6 h) for activated sludge under anoxic conditions. In both cases the experiments were performed at low initial E1 concentrations of 500 ng/l. However, when making a distinction for present data between a rapidly degrading fraction and a slowly degrading fraction, the specific k_{rapid}-value of 9.27 l.g $TSS^{-1}.d^{-1}$, derived from a k_{rapid} of 30.96 d^{-1} (Table 4.2), is approaching literature values.

Degradation of E1 under anaerobic conditions

The main process under anaerobic conditions is the reduction of E1 to E2, at a specific *k*-value of 0.033±0.012. l.g $TSS^{-1}.d^{-1}$ (Table 4.2). This observation was confirmed by Joss *et al.* (2004), although the process proceeded at a much higher rate, viz. 52±2 l.g $TSS^{-1}.d^{-1}$ for activated sludge and 60±15 l.g $TSS^{-1}.d^{-1}$ for MBR-sludge under anaerobic conditions.
In present research, the sum of E1 and E2 did hardly decrease over the entire experimental period (Figure 4.1C). However, Joss *et al.* (2004) reported a decrease of the sum of E1 and E2 under anaerobic conditions at specific *k*-values of 10±1 l.g $TSS^{-1}.d^{-1}$ ($t_{1/2}$=2 hrs) when using activated sludge and 28±3 l.g $TSS^{-1}.d^{-1}$ ($t_{1/2}$=19 minutes) with MBR-sludge, in both cases conducted at an initial concentration of 500 ng/l. These higher conversion rates could not be explained. The results from present research comply well with those of Kunst *et al.* (2002), who reported no decrease in the sum of E1 and E2 between 90 minutes and 10 days.

Results for the long-term anaerobic experiment are shown in Figure 4.4. The environmental conditions and the ammonium concentrations are summarized in Table 4.1. Also over a longer period, the sum of both compounds is hardly changing, indicating no net degradation under anaerobic conditions. The reduction of E1 to E2 is not always occurring to the same extent, Figure 4.4A and B only show a small amount of E2 was formed in digested pig manure and granular UASB sludge. In digested pig manure, the high ammonium

concentration (Table 4.1) might have inhibited this conversion. The absence of the reduction is unclear for the granular UASB sludge, but possibly due to its industrial origin (paper wastewater), the sludge did not develop E1 reducing properties. Similar findings with industrial sludge were reported by Layton *et al.* (2000), in which less than 4% removal of E1 and E2 was established in unexposed industrial sludge to 85% in activated sludge, both under aerobic conditions. The reduction of E1 to E2 is a biological process, as this reaction did not occur in the abiotic control. Czajka and Londry (2006) reported a production of E1 in E2 spiked sediments under methanogenic conditions. No E1 was produced in sterile controls. This indicates that E1 and E2 are biologically interconvertible under anaerobic conditions, possibly catalysed by other biological conversions.

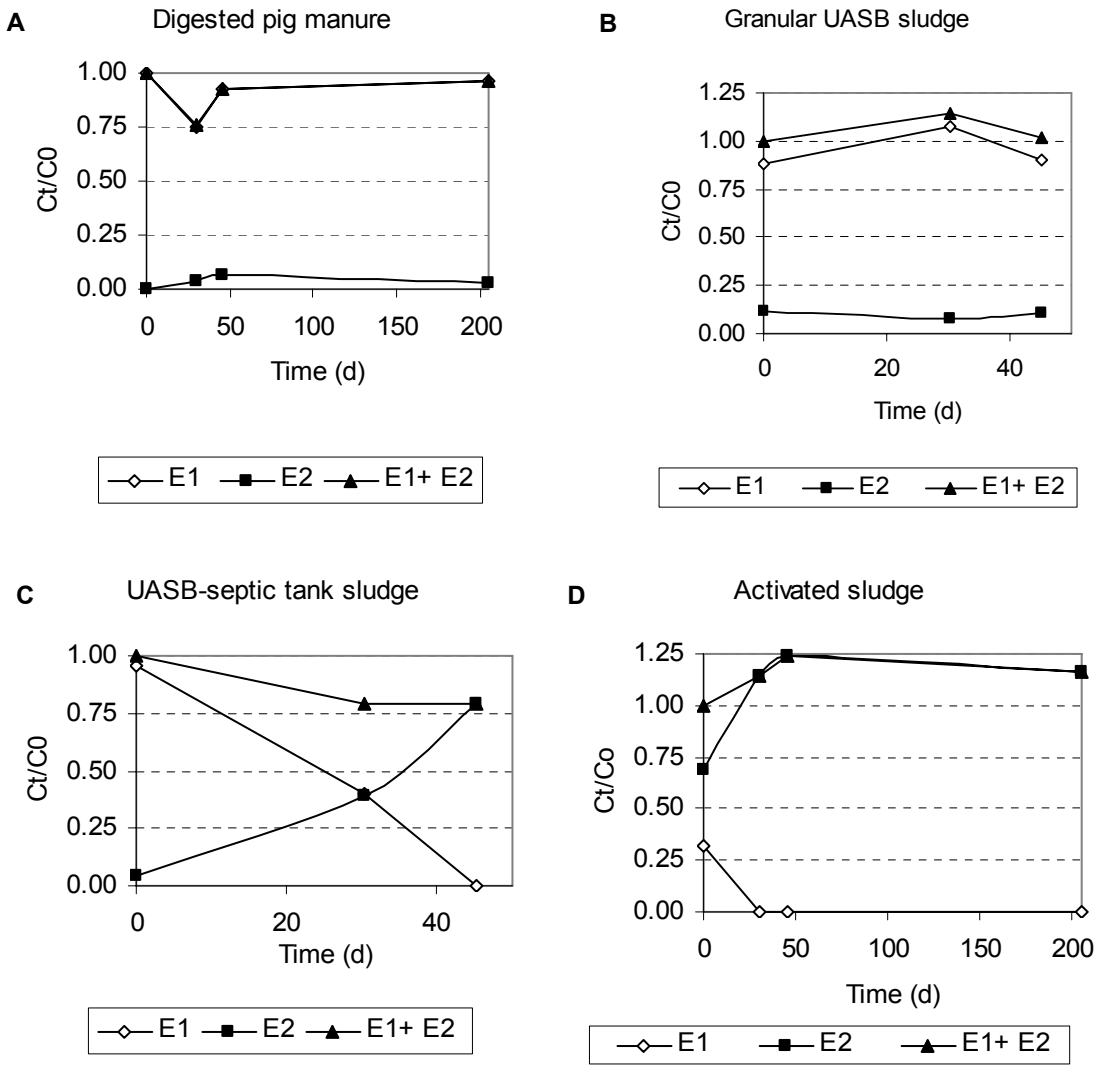


Figure 4.4. Conversion of E1 under anaerobic conditions at 30°C in (A) digested pig manure (B) granular UASB sludge (C) UASB septic tank sludge and (D) activated sludge.

4.3.2 Adsorption of E1 to activated sludge

Values calculated for the Freundlich adsorption coefficients of E1 to activated sludge (K_f and n_f) are presented in Table 4.4, together with values from literature. Figure 4.5 depicts the adsorption isotherm. The loss of E1 during the short-term adsorption experiment amounted to less than 10% after 1 hour contact. Similarly as found by Andersen *et al.* (2005), the value of n_f exceeded 1. According to Schwarzenbach *et al.* (2003) this would imply that the energy needed for sorption declines with higher amounts of the compound sorbed. Even though the sludge underwent freeze-drying and sterilisation in the quoted literature-case, similar K_f-values were found. The comparison between K_f obtained with undiluted and the K_f obtained with two fold diluted sludge (run C) reveals a higher adsorption of the compound per g VSS with diluted sludge. In the short-term adsorption experiments (run E), conducted under conditions of different E1 concentrations with the same amount of sludge, a lower K_f-value was found as compared to those calculated from the degradation tests.

Table 4.4. Calculated values for K_f and n_f in the aerobic degradation experiments run A, C, D and the short-term adsorption experiment E (with standard error and 95% confidence interval).

Run or reference	K_f ($l^n.mg^{1-n}.kg^{-1}$)	95% confidence interval		n_f (-)	95% confidence interval	
		lower value	upper value		lower value	upper value
E1-biodegradation experiments						
A	147.5±11.6	110.5	184.4	2.24±0.08	1.98	2.49
C	483.1±54.8	331.0	635.3	1.43±0.37	0.40	2.45
C +ATU	522.1±75.5	328.0	716.2	1.31±0.56	-0.13	2.74
C 2 x diluted	965.8±130.7	602.9	1328.6	1.25±0.6	0.25	2.25
E1-adsorption experiments						
E adsorption	100.7±74.4	81.4	282.7	4.14±0.91	1.92	6.35
Andersen *et al.* (2005)	89	-16	194	1.15	1.00	1.31
E2-biodegradation experiments						
D	125.6±13.0	98.7	152.5	0.20±0.03	0.31	0.27
E2-adsorption experiments						
Andersen *et al.* (2005)	1106	479	1733	0.77	0.68	0.86

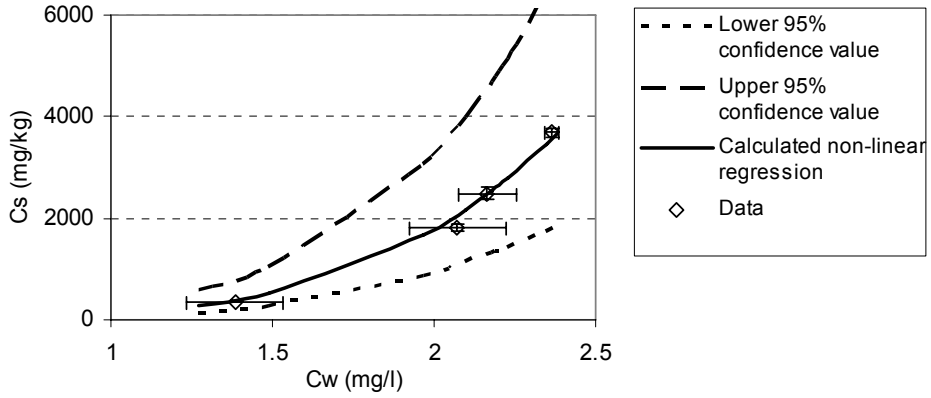


Figure 4.5. Adsorption isotherm, obtained from the short-term adsorption experiment (run E), including standard errors and the 95% confidence interval.

This higher K_f-value indicates that a.) during degradation a relatively large amount of the compound is still present in the sludge phase, and b.) that the re-establishment of equilibrium could proceed at a slower rate than the degradation of the compound from the liquid phase. Although direct evidence is unavailable, it does indicate that desorption is rate limiting.

4.4 Conclusions

- Biodegradation of estrone (E1) occurs at the highest rate with an average specific *k*-value of 0.27 l.g $TSS^{-1}.d^{-1}$ in activated sludge under fully aerobic conditions (DO= 8 mg/l), whilst under micro-aerobic conditions (DO= 1.3 mg/l) in activated sludge, its conversion rate is significantly lower with a specific *k*-value of 0.10 l.g $TSS^{-1}.d^{-1}$.
- Nitrifying organisms, present in activated sludge, do not contribute substantially in the degradation of E1.
- Under anoxic conditions, a low specific *k*-value of 0.004 l.g $TSS^{-1}.d^{-1}$ for degradation of E1 was obtained.
- Under aerobic conditions, the transformation of E2 to E1 proceeds 300-fold faster than the degradation of E1.
- Lower initial concentrations of E1 result in higher conversion rates.
- Under anaerobic conditions E1 is reduced to E2, but no decline in the sum of E1 and E2 did occur.
- No substantial decline in the sum of E1 and E2 was observed in a long-term anaerobic experiment in presence of digested pig manure, granular UASB-sludge, UASB-septic tank sludge and activated sludge.
- The rate and extent of E1 reduction in long-term anaerobic experiments did vary between the different types of sludge.
- Adsorption plays an important role in the removal of E1 from the liquid phase, with a K_f value of 101 $l^n.mg^{1-n}.kg^{-1}$.

Acknowledgements

The Dutch Economy, Ecology, Technology (EET) programme is acknowledged for the financial support of this project. The authors like to thank S.R. Stocking for the assistance in the preparation of the document.

Chapter 5

Determining key-parameters influencing the degradation of 17α-ethynylestradiol by sludge

Submitted for publication (De Mes *et al.*, in preparation)

Abstract

The fate of the synthetic estrogen 17α-ethynylestradiol (EE2) was investigated in various sludges with differing nature and under various redox conditions. EE2 is more persistent towards degradation than estrone (E1) and 17β-estradiol (E2) and also possesses higher estrogenic potency *in vivo* and *in vitro* tests. The key-parameter for degradation is the presence of oxygen. No degradation was observed in batch experiments under anoxic and anaerobic conditions, even when substrate was added and re-spiking was applied. No significant loss of EE2 occurred over a 256 day incubation period under anaerobic conditions after spiking digested pig manure, granular sludge of an upflow anaerobic sludge bed (UASB) reactor, flocculent sludge from a UASB septic tank, and activated sludge (AS). Moreover, even at low oxygen levels prevailing micro-aerobic conditions, a distinctly lower conversion rate of EE2 in AS was found as compared to full aerobic conditions, viz. first order degradation rates (*k*-value) of 0.014±0.003 d^{-1} and 0.077±0.005 d^{-1}, respectively. Except the imposed redox conditions, the nature of the sludge was found of importance; the highest *k*-value of 0.81±0.10 d^{-1} was obtained with membrane bioreactor (MBR) sludge. Also sludge originating from a sequencing batch reactor (SBR) gave a high *k*-value of 0.19±0.05 d^{-1}. From the results obtained using experiments with SBR sludge which did not contain nitrifiers (SRT <2 d) and those experiments conducted at inhibited nitrifying conditions in AS (*k*-value of 0.065±0.01 d^{-1}) it could be concluded that nitrifiers are not solely responsible for the degradation of EE2. High degradation rates (specific *k*-values) were obtained using sludge fractions with the smallest particles (<75 μm and 75-125 μm), viz. a value of 0.67 and 0.18 l.g $TSS^{-1}.d^{-1}$, compared to 0.029 l.g $TSS^{-1}.d^{-1}$ for the unfractionated AS. Adsorption experiments resulted in adsorption coefficients (K_f) of 567±86 $l^n.mg^{1-n}.kg^{-1}$ and 760±255 $l^n.mg^{1-n}.kg^{-1}$ for aerobic and anaerobic sludge respectively. At increased salinity (increased with 7.5 and 15 g/l) no significantly different K_f-values were found. Desorption probably influences the conversion rate of EE2, because the fraction that rapidly desorbs (F'_{rapid}: 0.58) is similar to the fraction that rapidly degrades (F_{rapid}: 0.51) as manifested in a two compartmental approach.

5.1 Introduction

Although the estrogenic potency of 17α-ethynylestradiol (EE2) *in vitro* and *in vivo* tests is higher compared to the natural estrone (E1) and 17β-estradiol (E2) (Purdom *et al.*, 1994; Routledge *et al.*, 1998; Legler, 2002), EE2 is present at a lower concentration. Moreover EE2 was found to be most persistent towards degradation in STPs (Belfroid *et al.*, 1999; Andersen *et al.*, 2003; Cargouët *et al.*, 2004), as confirmed in many batch tests performed with activated sludge under mainly aerobic conditions (Ternes *et al.*, 1999a; Layton *et al.*, 2000; Joss *et al.*, 2004; Shi *et al.*, 2004a/b). Most important removal processes of EE2 in STPs are adsorption, due to its high sorption tendency (log Kow 4.1 ;Lai *et al.*, (2000)), biodegradation and to a lesser extent photolytic degradation (Jürgens *et al.*, 2002).

Present research focusses on adsorption and degradation of EE2 by anaerobic as well as aerobic sludges under various redox conditions. The most important aim of present research is to determine the key-parameters and conditions affecting the degradation of this compound. According to literature data (Larsen *et al.*, 2004; Clara *et al.*, 2005), the sludge retention time (SRT) is an important factor for EE2 degradation; below a SRT of 5 days no degradation seems to occur in activated sludge. As nitrifying sludge develops at longer retention times, nitrifiers were expected to play an important role in the degradation (Vader *et al.*, 2000).

MBR sludge was reported to degrade EE2 faster compared to activated sludge. This has been attributed to the longer SRTs of the MBR sludge and/or by its different biological composition, as the complete sludge population is retained including colloidal and free cells (Joss *et al.*, 2004; Kjølholt *et al.*, 2004). When using MBR and activated sludges developed at similar SRTs, the degradation of EE2 was found to proceed at the same rate (Clara *et al.*, 2004b). In present research, experiments conducted with activated sludge were investigating the effect of aerobic, micro-aerobic, anoxic and anaerobic conditions, as well as the role of co-metabolism and re-spiking on the degradation of EE2. Applied redox conditions do occur in different zones of full-scale activated sludge STPs. Under aerobic conditions, the degradation of EE2 was also investigated in activated sludge under inhibited nitrifying conditions. The role of sludge nature on the aerobic degradation rate of EE2 was investigated using MBR sludge, cultivated at very long SRTs, and sludge from a sequential batch reactor (SBR) system, cultivated at a very short SRT. Under anaerobic conditions, a number of long-term experiments were conducted with different types of anaerobic sludges. Adsorption to anaerobic and activated sludge was investigated at different salt concentrations, as increasing salinity increased the adsorption of estrogens to estuarine sediments (Lai *et al.*, 2000; Bowman *et al.*, 2002; Braga *et al.*, 2005). The extent of mass transfer limitation was investigated in a desorption experiment with activated sludge, and by investigating the aerobic degradation rate of EE2 using activated sludge fractions with different particle sizes.

5.2 Materials and Methods

5.2.1 Experimental set-up

Biological degradation

Serum bottles (1-L) containing sludge, covered with aluminium foil to prevent photolytic degradation, were spiked with a stock solution of EE2 (CAS 57-63-6, Sigma-Aldrich) in HPLC-grade methanol (Acros, the Netherlands) to a concentration of 5 mg/l and kept at aerobic, micro-aerobic, anoxic and anaerobic conditions. Nitrification was inhibited by adding N-Allylthiourea (ATU, 20 mg/L). Anoxic conditions were ensured by addition of a stock solution of $NaNO_3$ (Merck, the Netherlands) at a rate of 14.4 mg N.g $VSS^{-1}.d^{-1}$. Aerobic batches were aerated, anoxic and anaerobic bottles were capped and flushed with N_2-gas after sampling. Additional research involved the addition of a second spike of EE2 and the addition of substrate, viz. acetic acid ($NaCH_3COO.3H_2O$ Acros, the Netherlands) at a concentration of 950 mg COD/l or artificial sewage (Berg and Nyholm, 1996). Activated sludge was sieved in five fractions, < 75 μm, 75-125 μm, 125-200 μm, 200-300 μm and > 300 μm, in order to assess the influence of floc-size on EE2 degradation.

The assessed recovery of EE2 in abiotic controls in AS with 0.5 g/l sodiumazide (Merck, Hohenbrunn) was 93.0±6.0% and with autoclaved AS (30 minutes at 121°C) 90.6±0.6%, both after 18d. All experiments were performed at ambient temperature, except for the degradation experiment with fractionated sludge and the anaerobic long-term experiments both at 30°C. The set-up is shown in Table 5.1 and 5.2.

Table 5.1. Experimental set-up of the degradation, adsorption and desorption experiments with EE2.

	Condition Degradation experiments					Adsorption	Desorption
Sludge origin	Aerobic	Aerobic nitrifying inhibition	Micro-aerobic	Anoxic	Anaerobic		
Activated sludge (AS)	A, B, C1, C2,D, F	C, F	C	B, E	B, C, E	K	M
MBR sludge (MBR-S)	G, H, I						
SBR sludge (SBR-S)	F						
Digested Pig manure (PM)					J		
Flocculent UASB septic tank (UASB-S)					J	L	
Granular sludge UASB (GS)					J		

Sludge origin

The activated sludge (AS) used in the experiments originated from the full scale oxidation ditch of Bennekom, the Netherlands, which is operated at an average SRT of 30 d. Membrane Bioreactor sludge (MBR-S) originated from a pilot plant treating the final effluent of the STP of the city of Leeuwarden, the Netherlands. The investigated MBR sludge samples were collected at different times, viz. the first sample was taken after 1.4 years of operation, the second sample was taken 4 months later. No sludge was discharged since start-up. The last sludge sample was taken one year after the first sample, the MBR was operated under a different feeding regime by the addition of an external C-source during the last six months of that year. Sequencing batch reactor sludge (SBR-S) was obtained from a pilot scale reactor operated in the experimental hall of the department of Environmental Technology, fed with raw influent from the STP of Bennekom, at an SRT of 1-2 days. The sludge exhibited no nitrifying capacity as was confirmed in a respiration test on a RA1000 (Spanjers and Keesman, 2002) (Appendix III). All aerobic sludges were sieved (2 mm) and aerated overnight prior to use. The anaerobic sludge samples used in the experiments were: a) digested pig manure (PM) from a full scale manure digester located in Sterksel, the Netherlands, b) granular sludge (GS) from a full scale UASB treating wastewater form the paper industry, Eerbeek, the Netherlands, c) flocculent sludge from a pilot-scale UASB-septic tank fed on black water (UASB-S)(Kujawa-Roeleveld *et al.*, 2005; Chapter 6) and d) activated sludge (AS) from the STP of Bennekom.

Adsorption

Adsorption experiments are performed in duplicate at four different concentrations of EE2, 2.5, 5.0, 7.5 and 10.0 mg/l, and at three different salt concentrations, achieved by adding artificial urine (Urmenyi *et al.*, 2005)(0%, 37.5% and 75%, equal to an increase in salinity to 0, 7.5, and 15 g/l). EE2 was measured in both liquid and solid phase after one hour stirring at temperature-controlled conditions of 25 ºC. To prevent a change in the structure, the sludge was not subjected to a deactivation procedure. The experiment is performed with AS and UASB-S sludge samples, both sieved prior to use (pores 2 mm). Non-settleable colloidal material in UASB-S was separated by centrifugation at 4000 rcf during 10 minutes (centrifuge IEC CL3); the supernatant was discarded and the solid fraction used.

Table 5.2. Experimental set-up of the degradation, adsorption and desorption experiments with EE2, BIO: biodegradation experiments, LT:long-term anaerobic experiment, AD: adsorption experiments, DES: desorption experiment.

Experiment	Sampling times (d)	Remarks
BIO-A	0.0, 0.1, 3.2, 5.2, 11.1,18.1, 24.1, 33.0	anoxic and anaerobic batches till t=18.1 hrs
BIO-B	0.0, 0.1, 0.9, 3.0, 5.8, 14.8	
BIO-C1	0.0, 1.0, 2.9, 8.9, 13.9,15.8, 21.8, 27.8, 34.8	3-L serum bottles
BIO-C2	0.0, 0.9, 2.9, 6.9, 12.8, 20.0, 31.0	Effect of second spike and substrate (artificial sewage[a]), 25 mg BOD.l-1.d^{-1}
BIO-D	0.0, 0.9, 9.0, 13.8	Different sludge fractions, 250 ml flasks, 30°C
BIO-E	0.0, 1.0, 3.8, 10.8, 17.9, 20.8, 25.8, 32.0	Effect of substrate ($NaCH_3COO$), effect of second spike
BIO-F	0.0, 1.0, 3.0, 7.1, 14.9, 21.9, 27.9	
BIO-G	0.0, 0.7, 1.1, 3.1, 5.1, 13.9, 20.7	
BIO-H	0.0, 0.3, 1.0, 4.0, 6.0, 13.0, 20.9, 24.9, 32.2	SRT effect, compared to G
BIO-I	0, 6.5, 24.0, 72.0, 144.75, 336.8, 505.0	SRT effect, operation change in MBR, addition C-source
LT-J	0.0, 30.2, 45.1, 205.3, 256.1	Long term experiment (250 d)
AD-K	1 hour	Adsorption experiment, effect of salt concentration, adding of artificial urine[b]
AD-L	1 hour	Adsorption experiment, effect of salt concentration
DES-M	0.0, 1.0, 1.8, 2.5, 3.3, 5.3, 7.3, 10.3, 13.3 (hrs)	Desorption experiment with Tenax

[a] Artificial sewage: peptone 16 g/L, beef extract 11 g/L, urea 3 g/L, NaCl 0.7 g/L, $CaCL_2.2H_2O$ 0.4 g/L, $MgSO_4.7H_2O$ 0.2 g/L (Berg and Nyholm, 1996). [b] NH_4HCO_3: 22.59 g/l, Na_2HPO_4: 0.68 g/l, $CaCl_2$: 0.14 g/l, K_2SO_4: 0.84 g/l, NaCl: 4.68 g/l, KCl: 2.24 g/l; total salinity 31.17 g/l (Urmenyi *et al.*, 2005).

Desorption

A desorption experiment is conducted in triplicate with the solid sorbents Tenax TA® (60-80 mesh: 177-250 μm, Bruchem BV Apeldoorn, the Netherlands) according Cornelissen *et al.* (1997). A 1-L serum bottle containing AS, spiked with EE2 in a concentration of 5 mg/l, was stirred during one hour (Hieroglyph, MR3001K, controlled at 20°C). After one hour, 40 ml sample was taken for analyses of EE2 in both the liquid and solid phase. Furthermore 3 samples of each 50 ml were taken and placed into a separation funnel with addition of 1 g of sodiumazide (NaN_3, Merck Hohenbrunn) and an excess of Tenax (1 g; the affinity of Tenax, is similar as for organic carbon, based on the current VSS 0.1 g is sufficient for 50 ml sludge mixture). The sludge mixture was horizontally shaken for pre-set times (Δsample times, Table 5.2) at 150 strokes per minute on a Gerhardt shaker in a temperature controlled room at 20°C. The Tenax was separated from the sludge mixture, and then extracted with methanol:acetone (vv. 1:1, 3 times 10 ml). The extract did undergo the same treatments as for the determination of EE2 in sludge as described below. To the sludge mixture again 1 g of fresh Tenax was added, and the procedure above described was repeated. Latter was completed another six times, so fresh Tenax was added a total of eight times. At the end the remaining amount of EE2 in the sludge mixture was determined.

5.2.2 Analytical determination and data processing

Well-mixed samples of each batch were taken at set times (Table 5.2) and EE2 was determined in both the liquid and solid phase according the method described in Chapter 3 and in de Mes *et al.* (2006). After pre-concentration and cleaning-up, the samples were measured for their EE2 content on a HPLC with C18 chromosphere column (L=2x10cm, external d= 6mm;C18 pre-column L=1cm, external d=6mm; mobile phase=acetonitrile(60%)+demineralised water(40%); flow rate=0.4 ml/minute; sample volume=10ml) with fluorescence (extinction 230 nm, emission 310 nm, with a cut off filter of 305 nm) and UV detection (200 nm) in series. Due to remaining liquid in the solid phase after centrifugation, an overestimate of 4.8±0.1% EE2 is calculated in the solid phase, corresponding to a maximum of 0.24 mg/l. In the anaerobic long term experiment, mixed liquor samples (20 g) were freeze dried, after which they underwent the same procedure as the sludge samples. The experimentally found limit of quantification (LOQ) is 10 µg/l in a sample, and values of 1 µg/l could still be detected (LOD).

Monitoring of pH, redox potential, O_2 concentration and temperature was done throughout the test period using portable meters (WTW). The total and volatile suspended solids (TSS and VSS) content was assessed at the beginning and termination of the experiments, using the 2540D/2540/E standard method (APHA/AWWA, 1998). In the anaerobic long term experiment TS and VS concentrations were measured according standard method 2540B/2540E (APHA/AWWA, 1998). To confirm the occurrence of denitrification in the anoxic batch and inhibition of nitrification in the aerobic batch with ATU, ammonium, nitrate and nitrite were measured in paper-filtered samples (Schleicher & Schuell, 5951/2 folded filters) at the beginning and end of the experiment, using a Skalar auto-analyzer according to ISO 11732 and ISO 13395.

First order degradation rates, *k*-values, (Equation 7, Chapter 2) and the 95% confidence intervals were calculated with non-linear regression in the statistical computer programme SPSS version 12.01. The specific *k*-value (l.g $TSS^{-1}.d^{-1}$) has been calculated using the TSS at the start op te experiment. Adsorption is described using the Freundlich isotherm (Equation 3, Chapter 2); both the adsorption coefficient and constant were calculated. A two compartmental approach was chosen (Equation 1) to calculate the fractions and the rates for rapid and slow desorption (Cornelissen *et al.*, 1997). Equation 2 in Chapter 4 is used to calculate the aerobic degradation rate of two different fractions for comparison with values obtained for the desorption.

$$\frac{C_t}{C_0} = F'_{rapid} \cdot e^{-k_{rapid} \cdot t} + F'_{slow} \cdot e^{-k_{slow} \cdot t} \qquad \text{Equation 1}$$

in which:

F'_{rapid} : fraction that is desorbing at a rapid rate (-)

F'_{slow} : fraction that is desorbing at a slow rate (-)

k'_{rapid} : rapid rate for desorption (d^{-1})

k'_{slow} : slow rate for desorption (d^{-1})

In order to compare results of experiments performed at 30°C with those conducted at ambient temperature, *k*-values are corrected for temperature using the Arrhenius equation (Equation 2).

$$\ln\frac{k_2}{k_1} = \frac{E}{RT_1T_2}(T_2 - T_1) \qquad \text{Equation 2}$$

In which T is the temperature in K, E is a constant characteristic of the reaction J/mol, R= ideal gas constant, 8.314 J/mol.K.
Equation 2 can be simplified to Equation 3:

$$\frac{k_2}{k_1} = \theta^{(T_2 - T_1)} \qquad \text{Equation 3}$$

Based on literature values for the degradation of EE2 in AS at two different temperatures, the calculated θ is 1.05 (Layton *et al.*, 2000), which complies with typical values reported for activated sludge systems, viz. between 1.02 and 1.10 (Metcalf and Eddy, 2003). A 1.53 times higher *k*-value is expected at 30°C as compared to prevailing average ambient temperature conditions of 22- 23 °C.

5.3 Results and Discussion

5.3.1 Biodegradation of 17α-ethynylestradiol

All calculated first order degradation rates (*k*-values) and the calculated specific *k*-values (l.g $TSS^{-1}.d^{-1}$) are illustrated in Figure 5.1. Significant differences between the calculated (specific) *k*-values in the different types of batch experiments can be derived on the basis of their 95% confidence interval. Figure 5.1 shows the highest first order degradation rate under aerobic conditions for MBR-S (third sludge sample, experiment I), followed by SBR-S; both are significantly higher than AS. The lowest rate was obtained with AS under micro-aerobic conditions, and no degradation was shown under anoxic and anaerobic conditions. When considering the specific first order rates, the highest value was calculated for fractionated AS, in the fraction containing the smallest particles, under aerobic conditions. The conversion of EE2 under every experimental condition is discussed separately below. The environmental parameters measured in the different experiments are summarised in appendix IV and the nitrogen mass balance for the anoxic batches is given in Appendix V. In appendix VI, the raw data of Figure 5.1 are given, including their standard errors, the measured TSS at the beginning of the experiment and the calculated half-lives. Appendix VII shows the outcome of a two-compartmental approach to degradation, viz. the fraction F_{rapid}, degraded according to k_{rapid} and F_{slow}, degraded according to k_{slow}.

Degradation of EE2 in AS under aerobic and inhibited nitrifying conditions

The results in Figure 5.2A show the course of the degradation of EE2 in AS under aerobic nitrifying conditions in time. The calculated specific *k*-value for the degradation of EE2 in AS is 0.029 l.g $TSS^{-1}.d^{-1}$(half-life 23.9 d/g TSS). Compared to values mentioned in literature,

which range from zero (no degradation) (Ternes *et al.*, 1999; Shi *et al.*, 2004b) to values up to 8±2 l.g $SS^{-1}.d^{-1}$(Joss *et al.*, 2004) in AS under aerobic conditions, values from present research correspond best with those of Shi *et al.* (2004a), who reported a specific *k*-value of 0.31 l.g $SS^{-1}.d^{-1}$ at an initial EE2 concentration of 1.0 mg/l. At a lower EE2 concentration of 0.2 mg/l but equal sludge load, they found a distinctly higher *k*-value, i.e. of 2.02 l.g $SS^{-1}.d^{-1}$.

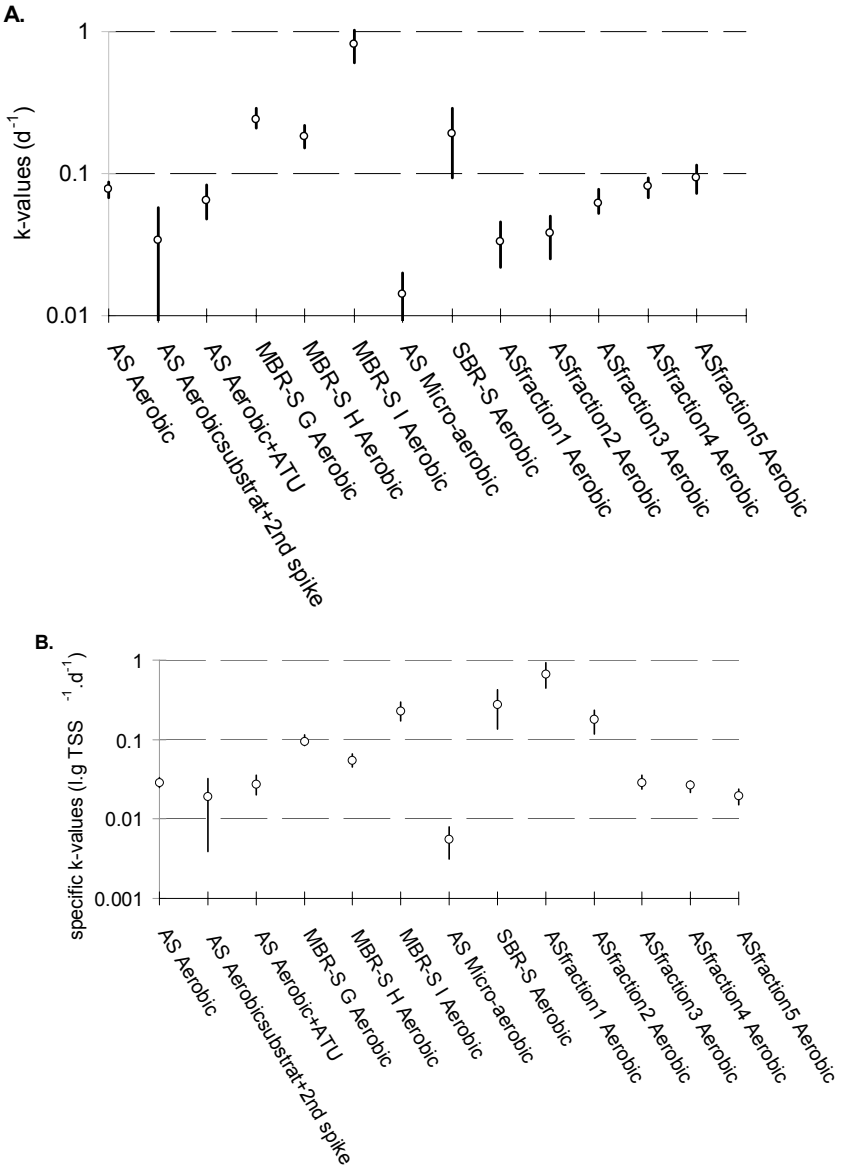


Figure 5.1. A: The 95% confidence interval of calculated first order degradation rates, *k*-values, for EE2 degradation under ambient temperatures for different sludges for imposed condition(s). B: The 95% confidence interval of calculated specific first order degradations rates, specific *k*-values, for EE2 degradation under ambient temperatures for different sludges for imposed conditions.

Calculated specific *k*-values in present research also correspond well with those of Layton *et al.* (2000), who found a *k*-value of 0.13 l.g $SS^{-1}.d^{-1}$ at an initial concentration of 72 µg/l, although they specifically looked at the mineralization rate of EE2 into CO_2, whereas in this research only removal of EE2 was assessed. Ternes *et al.* (1999) did not observe any removal of EE2 with an AS with a SRT <4 days, but once the plant was upgraded to nutrient removal

conditions, viz. operated at a SRT between 11 and 13 d, an elimination of 90% was attained (Andersen *et al.*, 2003). The reason for the absence of any degradation in the experiments of Shi *et al.* (2004b) could be due to the high initial concentration of 20-25 mg EE2/l. According to Kozak *et al.* (2001) inhibition occurs at EE2 concentrations exceeding 10 mg/l, except when fungi are used for degradation (Shi *et al.*, 2002). The highest degradation rates were found at low initial EE2 concentrations, viz. 8±2 l.g $SS^{-1}.d^{-1}$ at 100 ng/l (Joss *et al.*, 2004) and 3.03±1.21 l.g $SS^{-1}.d^{-1}$ at an initial EE2 concentration of 500 ng/l (Kjølholt *et al.*, 2004). Vader *et al.* (2000) reported a specific *k*-value of 0.6 l.g $SS^{-1}.d^{-1}$ at a concentration of 50 µg/l and Kikuta and Urase (2003) a specific *k*-value ranging from 0.12-0.96 l.g $SS^{-1}.d^{-1}$.

Under inhibited nitrifying conditions, the *k*-values and specific *k*-values remain very similar to those found under non-inhibited conditions (Figure 5.1). However, Shi *et al.* (2004a) reported 3 times slower degradation under inhibited compared to non-inhibited conditions and even demonstrated the ability of the nitrifier *Nitrosomas europea* to convert EE2 in a pure culture. The present results demonstrate that nitrifiers are not solely responsible for the degradation of EE2 in AS.

At the termination of experiment C, artificial wastewater and a second spike of EE2 was supplied to the batch to research the effect of addition of co-substrate and a second spike. Even though a slight increase of the VSS content was observed from 1.29 to 1.35 g/l, indicating some bacterial growth, the degradation of EE2 proceeded significantly slower compared to unfed conditions in which the VSS concentration decreased in time.

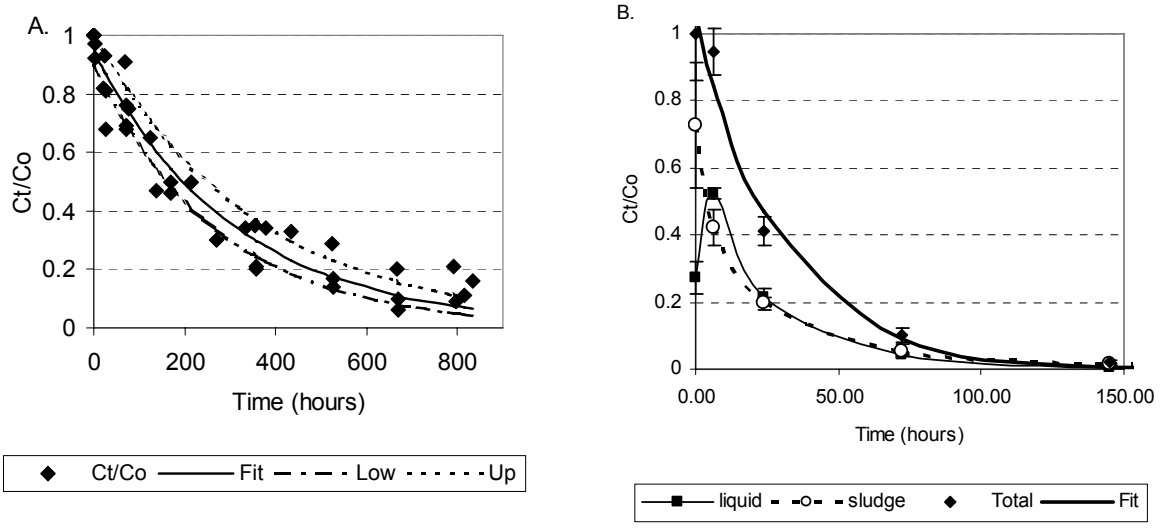


Figure 5.2. A: Degradation of EE2 in AS under aerobic conditions, with first order fit and 95% confidence interval. B: Degradation of EE2 in MBR-S under aerobic conditions, distribution over liquid and sludge phase, with first order fit.

Degradation of EE2 in MBR-S under aerobic conditions

The results in Figure 5.1 show that the difference in degradation rate of EE2 between the first two investigated MBR sludge samples is insignificant (experiment G and H). The third investigated MBR-S sludge sample, taken once the feeding regime in the MBR pilot-plant

was changed by addition of an external C-source, gave significant higher overall and specific degradation rates. Moreover, with all three MBR-sludge samples the degradation rates were significantly higher than for AS. These observations are similar to those of Joss *et al.* (2004). They attributed the higher rates to the smaller particle size of MBR-S sludge compared to AS sludge, i.e. to mass-transfer rate differences. Regarding the specific conditions imposed to the MBR pilot plant, i.e. the system was fed with an STP effluent in which hardly any easy biodegradable substrate is present, a specific microbial population might have developed, capable of degrading persistent compounds.

Degradation of EE2 in particle size fractionated AS at 30°C

Figure 5.1 shows the specific *k*-value under aerobic conditions (after temperature correction) for AS-fraction 5 with the largest floc-size being similar to the value for unfractionated AS. The confidence intervals for the calculated *k*-values in Figure 5.1 do not show significant differences between AS fraction 1 and 2 and also not between the fractions 3, 4 and 5, but between these groups the differences are significant. The specific *k*-value of AS fraction 1 is by far the highest. Also, the specific *k*-values show a significant difference between fraction 1 and 2, and between fraction 4 and 5.

Degradation of EE2 in SBR-S under aerobic conditions

Degradation of EE2 in SBR-S without nitrifying capacity under aerobic conditions occurs at a significantly higher rate compared to AS sludge (Figure 5.1). From literature it was found that AS with a low nitrifying capacity has no ability to degrade EE2 (Vader *et al.*, 2000). The different conditions imposed to the reactor may have selected micro-organisms, e.g. fungi or yeasts, which are more efficient in EE2 degradation. One explanation can be the presence of lignolytic enzymes, excreted by some bacteria and fungi, i.e. laccase which has the ability to form isomers of EE2 in presence of oxygen (Junghanns *et al.*, 2005). Suzuki *et al.* (2003) reported a complete degradation of EE2 within 8 hours, under influence of laccase at an initial concentration of 2.96 mg/l. Another explanation can be an enhanced bioavailability of EE2 in SBR systems as a result of improved solubility by the presence of bio-surfactants. SBR-systems according to Cassidy and Hudak (2001) favour the production of bio-surfactants relative to CSTR systems. The results obtained with SBR-S show that a required minimum SRT for the ability of degradation of EE2 of 5 days as reported by Larsen *et al.* (2004) and Clara *et al.* (2005) is not applicable for all treatment techniques. Also, as SBR-S applied in our investigations has no nitrifying capacity, it shows again that nitrifiers are not dominating EE2 degradation.

Degradation of EE2 by AS under micro-aerobic conditions

The results in Figure 5.1 reveal a very slow degradation of EE2 under micro-aerobic conditions, which might be due to the fact that the oxygen concentration is very low. This also emphasizes the important role that oxygen plays in the conversion of EE2.

Fate of EE2 in AS under anoxic and anaerobic conditions

No degradation manifested under anaerobic and anoxic conditions, in the presence and absence of substrate and also not upon a second spiking of EE2. The compound could be fully

retrieved at any time during all the experiments (Figure 5.3), even in the long term experiment of 205 days, except in the AS batch. In case of AS, half of the amount of EE2 was eliminated from the system, which was probably caused by increased oxygen levels at termination of the experiment (DO≈3 mg/l), as the caps were not tightly fitted to allow biogas to escape. Joss *et al.* (2004) reported a specific *k*-value under anaerobic conditions of 1.5±0.5 $l.g^{-1}$ $SS.d^{-1}$, which does not deviate significantly from the abiotic control value, i.e. a specific *k*-value of 1±0.5 $l.g^{-1}$ $SS.d^{-1}$. This is also true for the anoxic degradation reported by Joss *et al.* (2004), because it amounted to 1.2±0.3 $l.g^{-1}$ $SS.d^{-1}$ in AS and 3±2 $l.g^{-1}$ $SS.d^{-1}$ in MBR-S. Kjøholt *et al.*(2004), reported a specific *k*-value of 0.17±0.17 $l.g^{-1}$ $SS.d^{-1}$ for the degradation of EE2 under anoxic conditions and 0.012±0.054 $l.g^{-1}$ $SS.d^{-1}$ for the abiotic control, which also indicates no significant degradation. In literature a study was found dealing with a continuous sludge digestion experiment, in which an EE2 removal of 85±5% and 75±15% was established under respectively mesophilic and thermophilic conditions (Carballa *et al.*, 2006). Removal percentages were based on calculated spiked and background concentrations in the influent and measured concentrations in the effluent. Carballa *et al.* (2006) revealed that the removal occurred after sludge adaptation, but no information was supplied on the length of the adaptation period.

5.3.2 Adsorption of EE2

The calculated sorption coefficients and constants are summarised in Table 5.4. It appears from this table that the K_f values for UASB-S and AS are not significantly different. The sorption of EE2 to AS did not, unlike that on sediments, significantly increase at increasing salt concentrations. Which is in line with the results of Yanamoto *et al.* (2003) who reported no significant change in sorption of E2 to humic and fulvic acid with increasing ionic strength from 0.15 to 0.7 M. The high concentration of inorganic matter in sediment compared to AS may cause the observed difference. Whereas in the case of sediment, the salting out effect. i.e. a decrease of solubility with increasing ionic strength manifests (Schwarzenbach *et al.*, 2003), the high organic content of sludge can prevent the effect to occur. Furthermore sludge can also be subject to a number of changes as a result of salt addition, i.e. change of the surface charge and formation of complexes that hinder the effect. The adsorption coefficient found under non-equilibrium circumstances during degradation experiments is higher than in the adsorption experiments. This indicates that a relatively large amount is sorbed on particles and therefore not readily available to be degraded. Figure 5.6 illustrates the K_f-values with their 95% confidence interval.

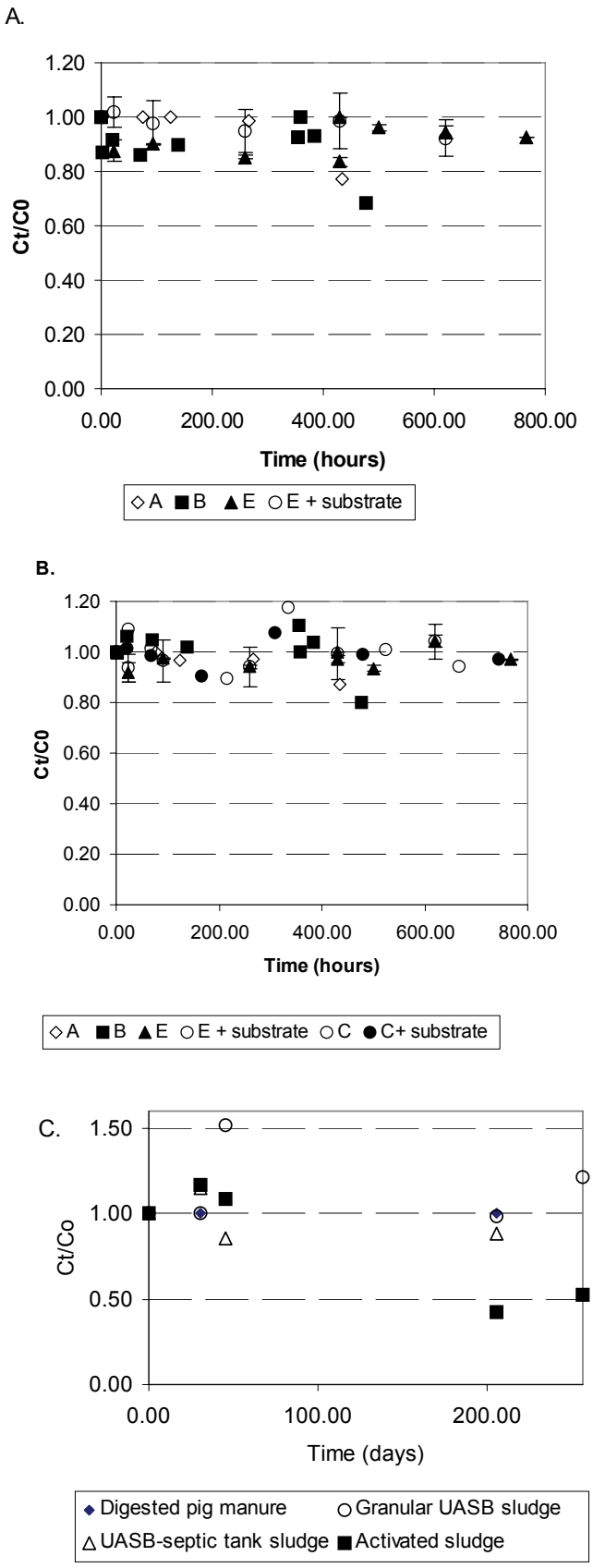


Figure 5.3. A: The concentration of EE2 in the AS-experiment under anaerobic conditions at ambient temperature. B: in the AS-experiment under anoxic conditions, and C: in the long-term anaerobic experiment with various types of sludge at 30°C.

Table 5.5. Adsorption coefficients (K_f) and constants (n_f) and their 95% confidence interval.

Experiment/reference	K_f	95% confidence interval		n_f	95% confidence interval	
	($l^n.mg^{1-n}.kg^{-1}$)	lower value	upper value	(-)	lower value	upper value
Values calculated from degradation experiments						
AS aerobic A, B, C and F	854±69	712	995	0.51±0.10	0.33	0.70
AS aerobic D fraction 3	1064±63	795	1333	0.66±0.03	0.51	0.80
AS aerobic D fraction 4	535±65	255	817	1.03±0.08	0.69	1.38
AS aerobic D fraction 5	744±46	545	942	0.75±0.05	0.55	1.00
MBR-S G,H	682±17	644	719	0.81±0.03	0.73	0.88
Values calculated from adsorption experiments						
Adsorption AS, salt 0	567±86	356	778	1.37±0.08	1.17	1.57
Adsorption AS, salt ¼	447±154	70	824	1.61±0.2	1.13	2.09
Adsorption AS, salt ½	878±165	473	1283	1.3±0.11	1.03	1.56
Adsorption UASB-S, salt 0	760±255	137	1383	1.0±0.2	0.5	1.5
Adsorption UASB-S, salt ¼	207±82	8	407	1.90±0.24	1.35	2.54
Adsorption UASB-S, salt ½	764±194	289	1240	1.2±0.17	0.77	1.62
Values reported in literature						
AS Ega STP (Kjølholt *et al.*, 2004)	267±257	n.a.	n.a.	1.08±0.12	n.a.	n.a.
AS Lundtofte STP (Kjølholt *et al.*, 2004)	383±245	n.a.	n.a.	1.02±0.09	n.a.	n.a.
AS (Clara *et al.*, 2004)	485	52[a]	559[a]	0.93±0.023	n.a.	n.a.
primary sludge (Ternes *et al.*, 2004)	278±3[b]	n.a.	n.a.	n.a.	n.a.	n.a.
secundary sludge (Ternes *et al.*, 2004)	344±37[b]	n.a.	n.a.	n.a.	n.a.	n.a.

[a]calculated with standard error from $\log K_f$. [b]K_d value (l/kg), instead of K_f, which is the simplified Freundlich: $C_s=K_d.C_w$ (Equation 4, in Chapter 2)

5.3.3 Desorption of EE2

Figure 5.6 shows the results of the desorption of EE2 with Tenax from sludge; this desorption represents a means to estimate the bioavailability of EE2 in the sludge, because an equilibrium between the sludge and Tenax is established. At t_0, 0.356 mg EE2 is present in 50 ml sludge mixture, of which 0.192 mg is present in the liquid phase and 0.164 mg EE2 in the sludge phase. At t_{end}, after 8 extractions with Tenax, 0.077±0.014 mg EE2 remains, comprising a fraction of 0.22 not being adsorbed to Tenax and this fraction can be seen, as being unavailable for degradation (Cornelissen *et al.*, 1997). The mass balance of the total amount of EE2 adsorbed to Tenax (0.216±0.03 mg) and the amount left shows a recovery of 89% relative to initial EE2-concentration present in the system, which implies that an average of 0.04 mg EE2 was lost, which is within the standard deviation range of 0.07 mg. Non-linear regression in SPSS gives a F'_{rapid} of 0.58 with a rapid desorption rate k'_{rapid} of 9.0 d^{-1} and a F'_{slow} of 0.42 with a k'_{slow} of 0.12 d^{-1}. The fractions F'_{rapid} and F'_{slow} for the desorption are similar to aerobic degradation of EE2, viz. 0.51 and 0.49 for F_{rapid} and F_{slow} respectively with a resembling k_{rapid} of 0.19 d^{-1} and k_{slow} of 0.041 d^{-1}. As completely different mechanisms are involved, the desorption and conversion rates can not be directly compared, the experimental desorption rates will always be higher compared to the degradation rates.

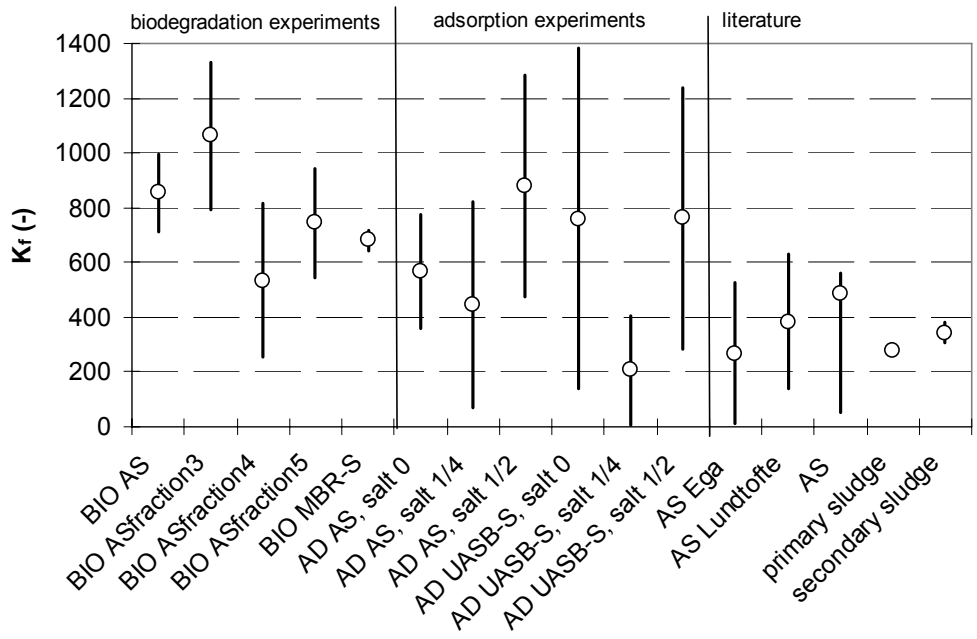


Figure 5.6. Calculated K_f-values with 95% confidence intervals during aerobic degradation experiments (BIO) and short-term adsorption experiments (AD) compared to literature values.

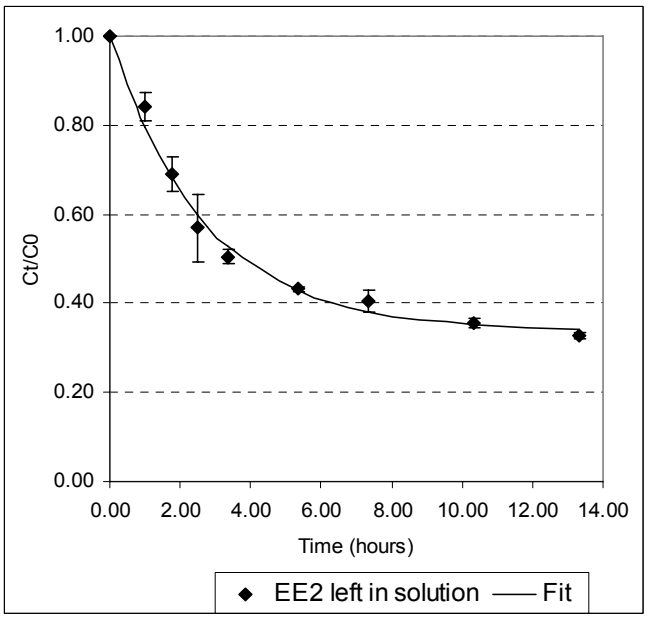


Figure 5.6. The assessed desorption isotherm of EE2 from AS using Tenax, i.e. the relative amount of EE2 remaining in the sludge mixture versus the shaking time; including standard deviation for the triplicate measurements at each t (real standard deviation at each t is the sum of the standard deviations of the times before).

However, when the rate of uptake, the metabolism and the mass transfer to the cell are optimised, degradation rates can approach the actual desorption rates in a biological treatment system (Bosma *et al.*, 1997). The similarity between the fractions desorbing and degrading on the rapid rate clearly indicate that conversion of part of the EE2 is limited by the desorption kinetics rather than the conversion rate.

5.4 Conclusions

- The key parameter for the degradation of EE2 in sludge is oxygen. Without oxygen, i.e. under anaerobic and anoxic conditions and at ambient temperature, degradation of EE2 was absent, even after re-spiking and addition of co-substrate.
- Specialised types of anaerobic sludge, viz. UASB-septic tank sludge, digested pig manure and granular UASB-sludge as well as activated sludge under anaerobic conditions, did not show any significant loss of EE2 after a 256 day incubation period at 30°C.
- Low oxygen levels, prevailing under micro-aerobic conditions, gave a lower conversion rate of EE2 in activated sludge compared to full aerobic conditions, viz. a first order degradation rate (*k*-value) of 0.014±0.003 d^{-1} and 0.077±0.005 d^{-1}, respectively.
- The nature of the sludge influences the degradation rate of EE2.
- Highest degradation rates for EE2 were observed in MBR and SBR sludge with *k*-values at ambient temperature of 0.81± 0.1 d^{-1} and 0.19±0.05 d^{-1}, respectively. The low SRT and absence of nitrification of the SBR sludge did not prevent EE2 degradation.
- Nitrifiers are not solely responsible for the degradation of EE2. Nitrification inhibition in activated sludge resulted in an EE2 degradation rate of 0.065±0.01 d^{-1}.
- Desorption probably influences the degradation rate of EE2 in sludge. The fraction EE2 that rapidly desorbed (F'_{rapid}: 0.58) is similar the the fraction that is rapidly degraded (F_{rapid}: 0.51).
- Adsorption plays an important role in the removal of EE2 from the liquid phase with adsorption coefficients (K_f) of 567±86 $l^n.mg^{1-n}.kg^{-1}$ and 760±255 $l^n.mg^{1-n}.kg^{-1}$for aerobic and anaerobic sludge respectively.

Acknowledgement

The research was performed in the framework of the "DeSaR-project" researching overall applicability of the concept of Decentralised Sanitation and Reuse, financed by the Dutch government under the EET programme. The authors would like to thank Tim Grotenhuis for his usefull comments, Simon Stocking for grammatical corrections and the Frysian Waterboard (Wetterskip Fryslân) for giving us the assignment of researching the ability of MBR sludge to degrade EE2 and their permission to publish the results.

Chapter 6

Fate of estrogens during anaerobic black water treatment with micro-aerobic post-treatment

Excerpt published in Proceedings Conference Advanced Sanitation, Aachen
de Mes T.Z.D., Zeeman G. and Lettinga G. (2007). Fate of estrogens during anaerobic black water treatment with micro-aerobic post treatment. Advanced Sanitation, Aachen.

Abstract

The fate of estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) was investigated in a concentrated black water treatment system consisting of an UASB septic tank, with micro-aerobic post-treatment. In UASB septic tank effluent, concentrations of natural total concentrations of 4.02 μg/l E1 and 18.69 μg/l E2, comprising the sum of conjugated (>70% for E1 and >80% for E2) and unconjugated forms, were measured. No EE2 was detected. In the effluent of the post-treatment E1 and E2 were present in concentrations of 1.37±1.45 μg/l and 0.65±0.78 μg/l, respectively. A percentage of 77% of the measured unconjugated E1 and 82% of E2 was associated with particles (>1.2 μm) in the final effluent implying high sorption affinity of both compounds. When spiking the UASB septic tank effluent with E1, E2, EE2 and the sulphate conjugate of E2, removal in the micro-aerobic post-treatment was >99% for both E2 and EE2 and 83% for E1. The lower removal value for E1 was a result of a combination of slow deconjugation during the treatment and with E1 being the first metabolite of E2 under aerobic conditions. In the final effluent still 40% of E1 and 99% of E2 was present in conjugated form. Latter was the result of incomplete deconjugation of the spiked E2(3S) in the post-treatment system.

6.1 Introduction

Two natural hormones estrone (E1) and 17β-estradiol (E2) and the synthetic hormone 17α-ethynylestradiol (EE2) are excreted by humans mainly in urine and a small amount in faeces. Therefore nearly all of these estrogens are present in black water when source-separation is applied. The estrogens in urine are mainly glucuronide or sulphate conjugates, formed to increase their solubility in water (Williams and Stancel, 1996). Although they do not exhibit any estrogenic potency in this form, microbial enzymes can cleave these conjugates back to their original active form. Sulphate conjugates are more stable than glucuronide conjugates and no decline in sulphate conjugates was observed in a septic tank for domestic wastewater (D'Ascenzo *et al.*, 2003).

Anaerobic biodegradation of estrogens is very slow or does not occur (Chapter 4 and 5) and up to 60% is adsorbed to sludge (Kunst *et al.*, 2002). Under anaerobic conditions, E1 and E2 are inter-convertible. Lee and Liu (2002) reported a 60% conversion of E2 into E1 after 20 days in activated sludge under anaerobic conditions at 21°C, spiked with E2 at an initial concentration of 2 mg/l. Similar findings were reported by Czajka and Londry (2006) for spiked lake sediment. Czajka and Londry (2006) researched anaerobic transformation of E2 under methanogenic, nitrate- , sulphate- and iron-reducing conditions. In all cases the sum of both E1 and E2 decreased with only 10% over 383 days.

All three compounds can potentially be removed under aerobic conditions (Ternes *et al.*, 1999a; Layton *et al.*, 2000; Chapter 4 and 5).

This chapter describes the results of research to the fate of E1, E2 and EE2 during anaerobic pre-treatment with micro-aerobic post-treatment of concentrated black water (Figure 6.1). The black water-stream is more concentrated than domestic wastewater or sewage, enabling implementation of anaerobic digestion for treatment and energy recovery. Since estrogens are excreted with urine and faeces, substantially higher estrogen concentrations are expected in black water when comparing to conventional sewage. The anaerobic pre-treatment was employed in an UASB-septic tank, which was demonstrated to

be a suitable reactor configuration for the treatment of concentrated black water (Kujawa-Roeleveld *et al.*, 2005). The Downflow Hanging Sponge (DHS) (Tandukar *et al.*, 2005; 2006) reactor was shown to be efficient in the removal of remaining COD and satisfactory for nitrogen removal from anaerobically treated domestic sewage. Besides, long employed sludge retention times may have a positive influence on the removal of estrogens (Clara *et al.*, 2005). This is demonstrated by higher removal percentages for E1 and E2 in a fixed bed, compared to an in parallel operated conventional activated sludge system, even though the hydraulic retention time (HRT) in the fixed bed was only ≈30 minutes, whereas ≥8 hours in the activated sludge system (Joss *et al.*, 2006). The removal percentage for EE2 was similar in both systems.

6.2 Materials and Methods

The research to the behaviour of estrogens during the different treatment steps for the treatment of black water consisted of two parts: (1) Measurements of "naturally" occurring E1, E2 and EE2 during anaerobic treatment and micro-aerobic post-treatment of concentrated black water and (2) Removal of E1, E2 and EE2 during micro-aerobic post-treatment of UASB septic tank effluent spiked with the estrogens E1, E2, EE2 and sulphate conjugate of E2(3S). Spiking was applied to get a better insight in the fate of all researched estrogens since no naturally occurring EE2 was detected in the system during the first part of the research.
The experimental set-up with indication of the sampling points is shown in Figure 6.1.

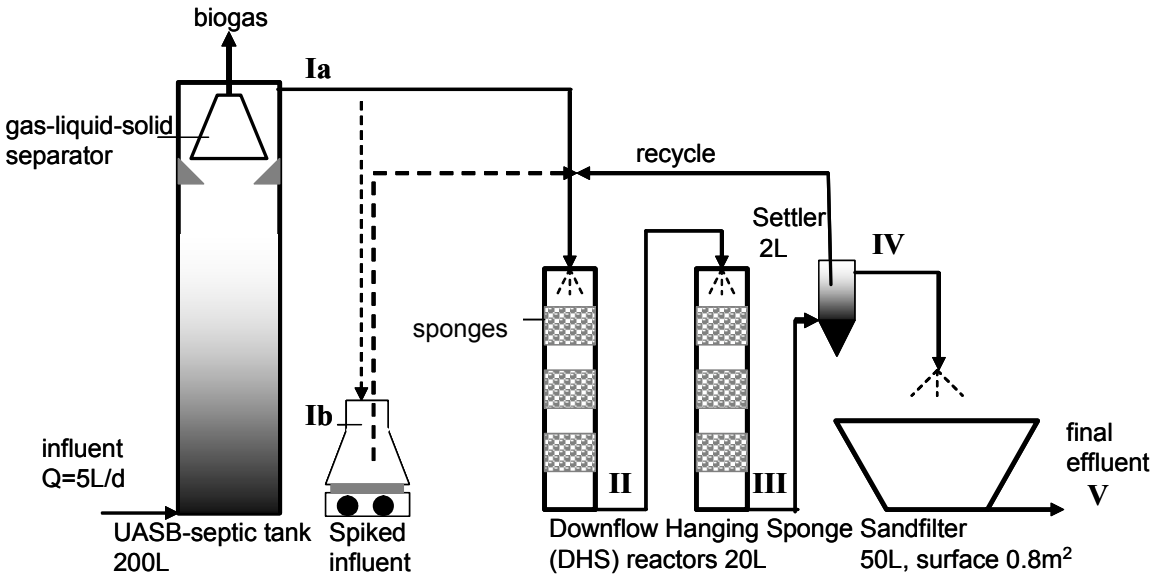


Figure 6.1. Black water treatment system. Sampling points Ia: UASB septic tank-effluent, Ib: spiked UASB septic tank effluent, II: DHS-1 effluent, III: DHS-2 effluent, IV: settler effluent (SET), V: sand filter (SF) effluent.

6.2.1 UASB septic tank pre-treatment

The 200 L UASB septic tank was designed for one person equivalent and black water was collected by vacuum toilets and people of the Sub-department of Environmental Technology

wre contributing till a weekly quote was reached. The used system is described in detail by Kujawa-Roeleveld *et al.* (2005). The UASB-septic tank was operated at an average hydraulic retention time (HRT) of 49 days and a sludge retention time (SRT) of 164 days (see also Table 6.2). The loading rate of 0.66 g.l reactor^{-1}.d^{-1} was similar as employed by Kujawa-Roeleveld *et al.* (2005). Measurements of natural occurring estrogens started after 401 days of operation, and 62 days before the effluent had been spiked with estrogens.

6.2.2 DHS reactors and sand filter post-treatment

The UASB-septic tank effluent was directed to the micro-aerobic post-treatment. Two cylindrical plexiglas DHS reactors contained three sponge sections each of 10 horizontally oriented sheets of reticulated polyurethane 10-20 mm thick foam-sponges (Recticel, Buren, the Netherlands), with the same diameter as the DHS, a specific surface area of 500 m^2/m^3, a density of 19-22 kg/m^3, 9-36 pores per cm^2 and a pore size of 2.5 mm. The distance between the sponge sections was 100 mm, the total height of the DHS reactor was 1 m with an inner diameter of 150 mm. Openings (d= 50 mm) located 200 mm above the bottom of the reactor ensured inflow of air. Recirculation (21 times the influent flow rate) was applied to guarantee a sufficient hydraulic load over the sponges.

The DHS was started-up with 10-L activated sludge from the low loaded activated sludge tank in RWZI Bennekom, the Netherlands. Seeding was established by distributing the sludge over the sponges via the influent inlet and recycling for 24-hrs. During a start-up period of 3 weeks, 10 times diluted UASB-septic tank effluent was applied as influent of the DHS; the following 3 weeks the dilution was gradually decreased to zero.

The sand filter, with a surface of 0.8 m^2, contained 25 kg gravel at the bottom and 25 kg of sand, divided into three layers. The first layer contained sand particles > 2 mm, followed by a layer of 0.5-1 mm and again > 2 mm at the top. After 296 days of operation, measurements in un-spiked wastewater commenced and 35 days before, the experiment with spiked UASB septic tank effluent was performed.

6.2.3 Sampling and analytical procedure for non-spiked effluent

The natural occurring estrogen concentrations in UASB-septic tank effluent (Ia) were determined during both research periods, once for only unconjugated forms and once for both, conjugated and unconjugated estrogens (in a grab sample prior to experiment with spiked UASB septic tank effluent).

E1, E2 and EE2 measurements commenced in semi-composite samples composed of 7 grab samples (200 ml) taken every other day over a period of 2 weeks, at sampling points Ia, IV and V (Figure 6.1). Three samples of 120 ml from the composite sample, referred to as samples A, B and C, were taken for analysis. The A and C samples were 400 times concentrated and the B samples 1200 times. The higher concentration was performed to ensure the estrogens to be available in the detectable range.

Recovery and matrix effects

In the C samples, a stock solution of E1, E2 and EE2 was added prior to the analytical clean-up and concentration steps, serving as identification of matrix effects on the recovery and detection. Besides, sample A was divided over two sample vials and in one series E1, E2 and EE2 (20 µl derivatised, 30.3 µg/l E1, 90.1 µg/l E2 and 41.5 µg/l EE2) was added to directly account for any matrix effects. Recovery was also determined in Millipore water spiked with 0.15 mg/l and 0.76 mg/l E1, 0.45 mg/l and 2.25 mg/l E2 and 0.21 mg/l and 1.04 mg/L EE2, which underwent the same treatment as the samples.

Procedure

Samples were filtered over a glass fibre filter (GF/C, Whatman), to prevent clogging of the Solid Phase Extraction (SPE) disks (SDB-X, Varian, the Netherlands). All filters were extracted with actetone:methanol (vv 1:1), and followed the same procedure as the filtrates. After SPE, an extra silica gel clean up was applied (Ternes *et al.*, 1999b). In order to increase the volatility, completely evaporated samples were derivatised for 1 hour at 60°C with 25 µL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MFSTFA; Sigma), containing 4 mg 1,4-dithioerythritrol (Merck) and 2 mg ammoniumiodide per ml. After derivatisation the fluid was evaporated under a gentle stream of N_2-gas and reconstituted in HPLC-grade petroleum ether (Suprasolv, Merck).

Volumes of 2 µl of the derivatised extracts or standard solutions were injected in a GC-MS Interscience system (Breda, the Netherlands) consisting of a Trace GC 2000 gas chromatograph equipped with a PTV injector operated in splitless mode, and AS 2000 sampler and a Polaris Q ion-trap mass spectrometer (Thermofinnigan, Breda, the Netherlands). Compounds were separated on a 50 m x 0.25 mm I.D. low bleed-MS column coated with a 0.25 mm film of CDP-Sil 8 CB (95% dimethyl-5% phenyl polysiloxane; Varian Chrompack, the Netherlands). Helium was used as carrier gas at a constant flow of 1 ml/min. The injector, ion source and mass transfer line temperature were 250°C, 250°C and 300°C respectively. The temperature programme almost similar as described in (Noppe, 2006) was used: initial temperature 30°C, first ramp with 30°C/minute to 130°C, second ramp with 2°C/minute to 268°C, on hold for 10 minutes, then the last ramp with 20°C/minute to 300°C and hold for 5 minutes to clean out the column. Data processing was performed using Xcalibur 1.4 software (Thermofinnigan, Interscience) using the molecular masses from Table 6.1. The calibration was based on a five point calibration curve. A detailed descritpion of the method is given in Chapter 3.

Table 6.1. General conditions during GC-MS analysis and quantification masses as determined in derivatised standards (MW: Molecular weight).

Compound	Retention time (min)	MW	MW derivates	*m/z* used for quantification
Estrone (E1)	28.80	270.4	414	218, 257, 342, 414
17β-estradiol (E2)	27.30	288.4	416	129, 285, 326, 401, 416
17α-ethynylestradiol (EE2)	30.90	296.4	440	285, 300, 425, 440

6.2.4 Sampling and analytical procedure for spiked effluent

UASB septic tank effluent was daily collected in an 8-L erlenmeyer, freshly spiked with estrogens (E1: 710 µg/l, E2: 1930 µg/l, EE2 2170 µg/l and E2(3S): 486 µg/l), placed on a magnetic stirrer (300 rpm, Heidolph MR3001K) and continuously pumped with a peristaltic pump to the micro-aerobic post-treatment. Prior to sampling, the plant was fed with spiked wastewater for 3 days. Semi-composite samples consisted of 8 samples, collected over the course of a day with the last one taken in the morning prior to refreshing the influent. The samples were collected during 3 days at sampling points Ib, II, III and V (Figure 6.1). Daily, 50 ml of a semi-composite sample was freeze-dried, extracted with acetone:methanol (vv 1:1, Acros) and concentrated by solid phase extraction (SPE) using C18 disks (Varian, the Netherlands). After complete evaporation of the extract under a gentle stream of nitrogen gas the sample was reconstituted in 1-ml methanol and measured on an HPLC with a C18 column of 2x10 cm (d=5 mm) and a mobile phase of acetonitrile (40%) with UV (200 nm) and fluorescence (extinction 230 nm; emission 310 nm, cut-off filter 305 nm). The method is extensively described in Chapter 3 and De Mes *et al.* (2006). Additionally, an enzyme solution (Helix promatia; Sigma) with glucuronidase and sulphatase was added to another 50 ml of the semi-composite samples according to Huang and Sedlak (2001) to determine the fraction of conjugated estrogen forms.

The pH, redox potential, O_2 concentration, temperature were monitored in the semi-composite samples using portable meters (WTW). Volatile fatty acids (VFA), total, particulate, colloidal and soluble COD (COD_{tot}, $COD_{SS,}$ COD_{col}, and COD_{sol}) were determined according to Elmitwalli (2000). Ammonium (NH_4-N), nitrate (NO_3-N), nitrite (NO_2-N) were measured according to ISO117732 and 13395 methods.

6.3 Results

6.3.1 Pilot-plant performance

The process parameters for the operation of the UASB-septic tank are summarized in Table 6.2. Stability of the system was confirmed by low effluent VFA concentrations over the whole operational period of 339 d. Obtained removal efficiencies for the UASB septic tank were similar as reported by Kujawa-Roeleveld *et al.* (2005). Recorded temperatures were on average 25°C in the UASB septic tank and 22°C in the micro-aerobic post-treatment. The redox potential was -356±57 mV in UASB-effluent and increased to 62±62 mV in the sand filter effluent. DO levels were ranging from 2.79±1.15 mg/l in the effluent of DHS1 to 3.05±1.19 mg/l in the effluent of the sandfilter. The pH was 8.40±0.20 in the UASB septic tank effluent, 7.67±1.19 in the effluent of DHS1, 7.36±1.54 in the effluent of DHS2 and 7.86±0.78 in the effluent of the sand filter.

After the first DHS, the particulate fraction of COD increased (Figure 6.2A) whilst the colloidal fraction decreased. Produced particulates in the first DHS were for a large part removed in the second DHS and almost completely retained in the sand filter. Only a small amount of colloidal COD was still present in the final effluent of the system.

The concentration of COD soluble did not significantly change over the whole treatment train. The observed brown colour of the effluent is amongst others caused by the presence of humic acids produced during biological treatment of black water (Gulyas *et al.*, 2004). The black water produced in current research is about 30 times more concentrated compared to combined municipal wastewater at dry weather conditions.

Table 6.2. Process parameters of the UASB-septic tank and effluent quality.

Parameter	
COD influent (g/l)[a]	29.2±7.7
Flow rate (l/d)	4.1±0.7
Organic loading rate ($g.l^{-1}.d^{-1}$)	0.66
HRT (d)	49
SRT (d)[b]	164±59
Total TS in UASB (g)	2413±161
Total VS in UASB (g)	1457±173
VFA effluent (g/l)	0.155±0.097
COD_{tot} effluent (g/l)	2,591±0.520
% COD removal	91

[a]Calculated on basis of recorded contributions in the vacuumtoilet; 1 x faeces (200 ml or 138 g): 78.3 g COD; 1 x urine (200 ml): 2.56 g COD, 1 x flushing: 0.5 L, and toilet paper is 13.5 g $COD.person^{-1}.d^{-1}$, measured influent values were 31.4 ±.3.4 g/L (n=3)

[b]Calculated of total amount sludge in UASB (g VS) divided by the amount discharged (g VS/d) plus the amount discharged with the effluent (g VS).

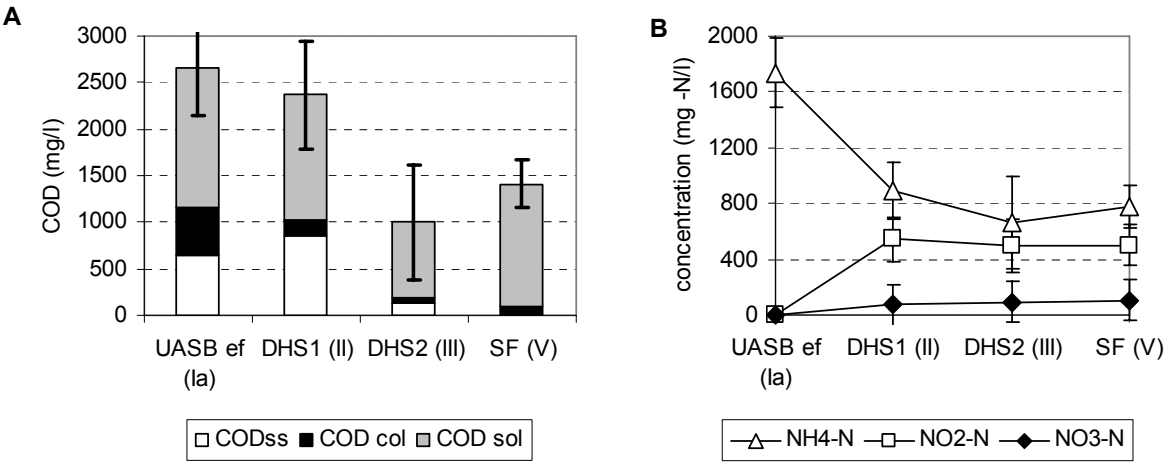


Figure 6.2. (A) COD fractions in the influent and effluents of the pilot plant for treatment of concentrated black water; error bars refer to COD_{tot}. (B) Concentrations of ammonium (NH_4-N), nitrate (NO_3-N) and nitrite (NO_2-N) in the influent and subsequent effluents of the pilot plant for treatment of black water.

The effluent soluble COD of treated black water (1325±241 mg/l) corresponds to the effluent values for activated sludge systems treating municipal wastewater, which is typically in the range of 30-50 mg/l under dry weather conditions, multiplied with factor 30 (which is the dilution factor) and can be considered as inert fraction (Roeleveld and Loosdrecht, 2002). Sixty percent of ammonium was nitrified (Figure 6.2B). Based on ammonium, nitrate and nitrite measurements over the DHS reactors a total N-removal by denitrification of 486±48 mg N/l was calculated.

6.3.2 Fate of E1, E2 and EE2 during micro-aerobic treatment of anaerobic pre-treated black water

The concentration of estrogens in the UASB-septic tank effluent (Ia) measured with HPLC were 1.23±1.80 μg/l for E1 without enzyme addition and 4.02±0.52 μg/l with the enzymatic deconjugation step and for E2 respectively 3.81±4.27 μg/l and 18.69±10.04 μg/l (Figure 6.3). Figure 6.4 shows the actual measured concentrations in the different sampling points. UASB-septic tank effluent values measured with GCMS were 2.80±1.66 μg/l for E1 and 1.00±0.28 μg/l for E2, comprised of the sum of unconjugated forms in both liquid and particulate phase. No EE2 was detected in any of the samples.

The fractions of conjugated E1 and E2 (70 and 80% respectively) are therefore significant.

In present research it was impossible to take composite samples from the black water influent, but the measured UASB-septic tank effluent value is in the range of the expected concentrations for total estrogens in concentrated black water, i.e. 21 and 42 μg/l when a low toilet flushing volume of respectively 1 and 0.5 l is applied (De Mes and Zeeman, 2003). In anaerobic long term batch experiments with anaerobic sludges of various origins no degradation of any of the three estrogens was observed (Chapter 4 and 5).

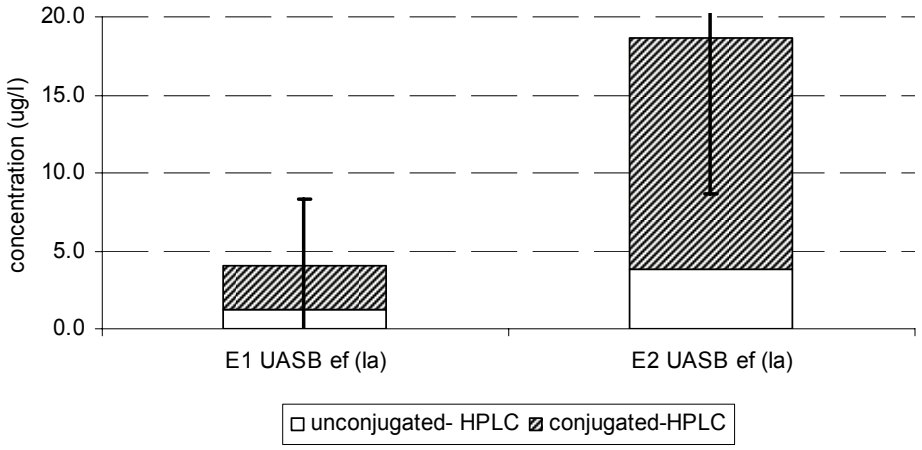


Figure 6.3. Concentrations of conjugated and unconjugated E1 and E2 in UASB septic tank effluent determined in freeze-dried samples by HPLC.

A treatment plant treating farm swine waste, consisting of a UASB (with HRT 2 days) and a trickling filter (with HRT of 1 day) showed similar concentrations of E1 and E2 in the UASB effluent measured on two occasions; 3.7±0.14 μg/L for E1 and 0.44± 0.04 μg/l for E2 (Furuichi *et al.*, 2006). Furuichi *et al.* (2006) also reported raw UASB influent values for swine wastewater, which were 5.3±0.14 μg/l for E1 and 1.25±0.35 μg/l for E2, which gives an average loss of 30% of E1 and 63% of E2 in the UASB. However it must be noted, that samples were filtered through a glass-fibre filter and that estrogens associated with particles >1.2 μm were excluded from determination, in both influent and effluent measurements. The effluent values for unconjugated E1 and E2 in the liquid phase of the DHS were 170 ng/l and 110 ng/l, respectively. The trickling filter effluent values reported by Furuichi *et al.* (2006),

were a lower compared to present research, but differ a lot between the two grab samples; 6.9 ng/l and 120 ng/l for E1 and <0.3 ng/l and 4.5 ng/l for E2.

Distribution over liquid and solid phase

A significant fraction of unconjugated E1 and E2 in the effluents of UASB septic tank, settler and a sand filter was associated with the colloidal and suspended material (particles< 1.2 μm). Figure 6.5 shows the distribution of unconjugated E1 and E2 over the liquid and the colloidal/suspended phase at the different sampling points. The fraction associated with particles is slightly higher for E1 as compared to E2. Over the treatment train, the relative amount present in the colloidal/suspended phase is increasing for both compounds. This indicates that the amount in the liquid phase is disappearing faster, which supports the hypothesis of desorption being the rate limiting step during the removal of estrogens as speculated in Chapter 4 and described in Chapter 5. The effect of limited bioavailability was also observed to be strong in soils; in soil spiked with ^{14}C radio-labelled E2, after 5 days a decline was observed solely in the extractable ^{14}C and the non extractable ^{14}C remained unchanged (Fan *et al.*, 2007).

The observed overall removal was approximately 50% for E1 and E2 over the post-treatment, based on measurements of unconjugated estrogens. In this experiment conjugated estrogens were not determined. Calculation based on the total influent concentrations (conjugated + unconjugated) removal percentages are 66% and 97% for E1 and E2, respectively. Removal percentages of 78% for E1 and 85% for E2 were observed for the removal over the micro-aerobic treatment based on the concentrations in the liquid phase.

The recoveries in Millipore water and in the standard A series to which a known amount of derivatised standard was added, were satisfactory as illustrated in Table 6.3. Also recoveries in the C-series were satisfactory for E1 for all the sampling points. Lower recoveries were observed for E2 (47%) and EE2 (11 and 17%), especially in the UASB-septic tank effluent. This indicates that concentrations in the UASB septic effluent are actually higher, so that the post-treatment system is operating more efficient as can be concluded from the measurements.

Table 6.3. Recoveries in spiked Millipore water, in separate A-series spiked with derivatised standard prior to analysis, and in C-series spiked with E1, E2 and EE2 prior to sample treatments.

% recovery	E1	E2	EE2
Spiked Millipore 1	84	129	80
Spiked Millipore 1	112	85	88
A- UASB Ia	97	70	85
A- Settler IV	107	75	94
A- Final effluent	109	76	95
C- UASB Ia	82	47	17
C- Settler IV	100	63	11
C- Final effluent	70	68	48

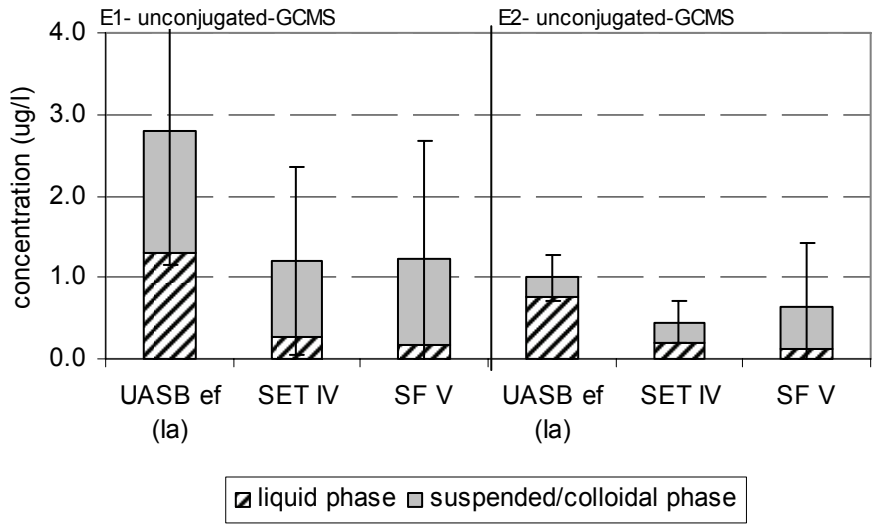


Figure 6.4. Concentrations of E1 and E2 measured over the pilot plant in the liquid and the colloidal/suspended phase determined by GCMS (standard deviations refer to the total values).

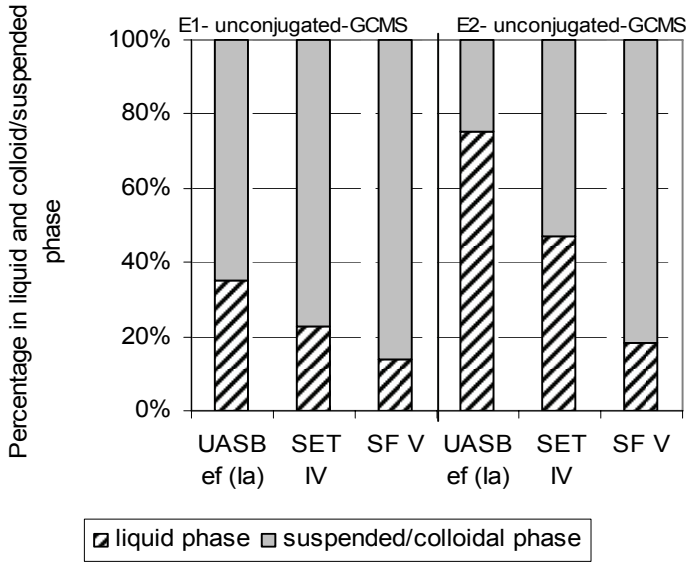


Figure 6.5. Distribution of unconjugated estrogens over the liquid and colloidal/suspended phase in samples taken at the different sampling points.

6.3.3 Fate of E1, E2, E2(3S) and EE2 during micro-aerobic post-treatment of spiked anaerobic black water effluent.

The measured concentrations of E2 and EE2 in the influent tank to DHS1 (Ib) after spiking with mentioned estrogens including a conjugated E2, were generally significantly lower as expected based on the spiked amounts (Figure 6.6A). Constant stirring in the influent storage tank probably introduced oxygen, which enhanced biodegradation. A depletion of 99 and 38% for E2 and EE2 respectively, was measured. The mean E1 concentration was 5% higher, probably as a result of conversion of E2 to E1. However, the depleted amount of E2 was substantially higher than the increased amount of E1. E1 was most probably also degraded in the influent storage tank. Besides, increased values of E1 and E2 in the influent are expected to be caused by cleavage of E2(3S) under (micro-)aerobic conditions.

The overall removal throughout the whole treatment plant was highest for E2 and EE2 (Table 6.3). The lower removal percentage for E1 can be explained by the slow deconjugation of the added E2(3S), for which a first order degradation rate of 0.28 d^{-1} was reported in wastewater in absence of sludge under continuous agitation at 20°C (D'Ascenzo *et al.*, 2003). This value is similar to the specific first order degradation rate of 0.27 l.g $TSS^{-1}.d^{-1}$ of E1 reported in Chapter 4 in presence of activated sludge. After the slow deconjugation of E2(3S) into E2 during the treatment, E2 was rapidly converted into E1 under aerobic conditions, before further conversion occurred (D'Ascenzo *et al.*, 2003; Chapter 4). These findings indicate that the presence of conjugates influences overall observed removal of estrogens. At present, little attention has been paid to the deconjugation rate under different redox conditions, which deserves more attention in future research.

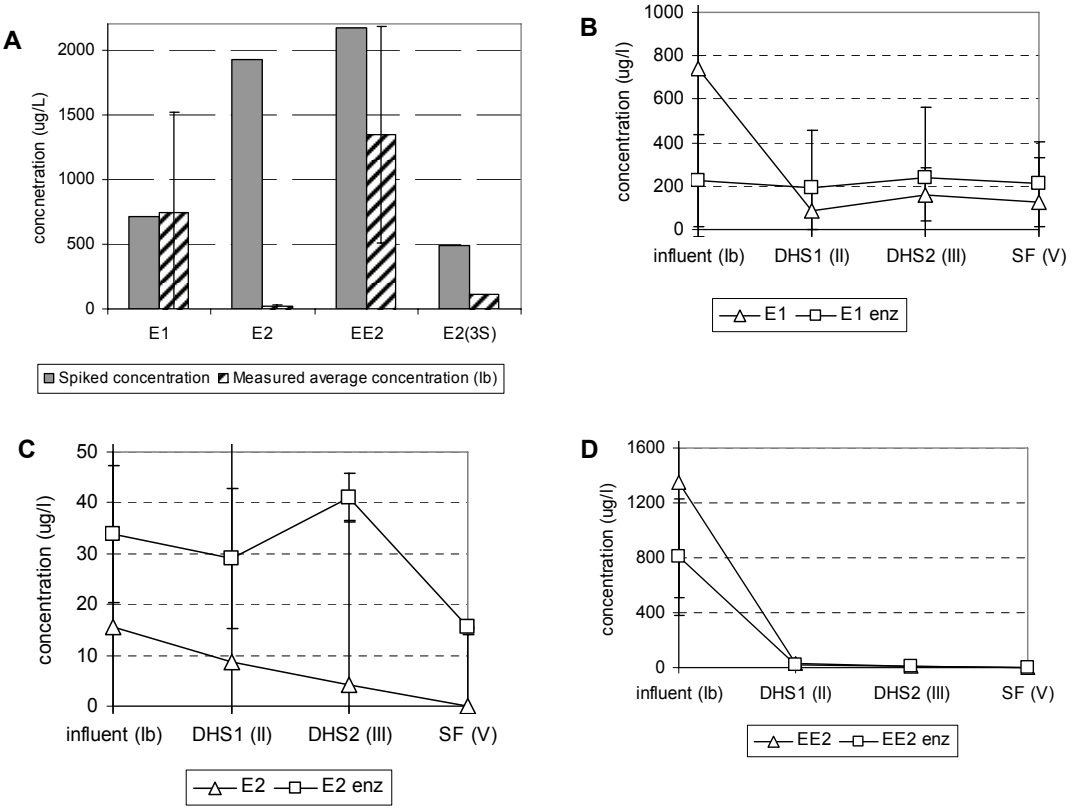


Figure 6.6. (A) Spiked concentrations and measured average estrogen concentrations in the influent tank (Ib) of the micro-aerobic post-treatment without enzyme addition; E2(3S) calculated based on difference between measurement in sample with and without enzyme addition corrected for loss during enzymatic reaction (70% for E1 see chart B); Measurements of: (B) E1 and E1 enz (enz: including enzymatic deconjugation) (C) E2 and E2 enz and (D) EE2 and EE2 enz in the pilot plant fed with spiked influent.

A lower E1 removal percentage was also reported for conventional secondary treatment; Drewes *et al.* (2005) reported a removal of 85, 98 and 94% for the removal of E1, E2 and EE2 respectively. Also for conventional treatment systems with activated sludge systems in different European countries, E2 was only detected in 5 out of 16 STPs in a concentration of 2.7±1.9 ng/l whereas E1 was detected in 13 out of 17 STPs in a concentration of 3.7±2.8 ng/l (Johnson *et al.*, 2005). Fourteen pure cultures, isolated from activated sludge, were all able to

convert E2 into E1, whereas only three were able to degrade E1 of which only one was able to use E2 as a single carbon source (Yu *et al.*, 2006). In a lagoon constructed-wetland system treating swine wastewater, E1 also showed to be the most persistent natural estrogenic compound (Shappell *et al.*, 2006).

As follows from Figure 6.6B, C and D, the addition of enzymes to the 50 ml samples before analysis, for deconjugation of conjugated estrogens, did not always result in higher total estrogen concentrations. This does not automatically imply that no conjugated estrogens were present, but as the enzyme-solution was added to unfiltered samples, degradation during its 20 hours reaction time at the recommended temperature of 37°C can not be excluded. In the final effluent of the sandfilter, the conjugates contribution was at least 40 and 99% for E1 and E2 respectively (Figure 6.6B, C and D). The high value for E2 indicates an incomplete deconjugation of the stable E2(3S) conjugate over the post-treatment system.

Table 6.3. Removal of estrogens (in %) over the different treatment steps and overall removal over the whole post-treatment pilot plant of spiked and naturally present estrogens.

	Spiked E1	Naural	Natural E1	Spiked	Naural	Natural E2	Spiked
DHS1 (II)	89	n.a.	n.a.	44	n.a.	n.a.	98
DHS2 (III)	-90	n.a.	n.a.	51	n.a.	n.a.	73
SET (IV)	n.a.	69	67	n.a.	97	72	n.a.
SF (V)	22	-8	37	97	-46	42	86
overall removal	83.1	66	78	99.2	97	85	99.9

6.4 Conclusions

- Only E1 and E2 were detected in anaerobic pre-treated black water, E2 being dominant;
- E2 and EE2 were removed to a high degree (>99%) from spiked UASB septic tank effluent during the micro-aerobic post-treatment consisting of two DHS reactors and a sandfilter in series. E1 was removed for 83%.
- The unconjugated natural occurring amounts of E1 and E2 in liquid plus adsorbed to particles, were respectively 2.80±1.66 μg/l and 1.00±0.28 μg/l after anaerobic treatment and 1.37±1.45 μg/l for E1 and 0.65±0.78 μg/l in the final effluent of micro-aerobic system.
- The largest part of the naturally occurring E1 (>70%) and E2 (>80%) in UASB septic tank effluent was present in the conjugated form; in the final effluent after micro-aerobic post-treatment of spiked UASB effluent, respectively 40% of E1 and 99% of E2 were present in conjugated form.
- In the UASB septic tank effluent 53% of natural measured unconjugated E1 and 25% of E2 was associated with particles larger than 1.2 μm. In the final effluent of the sandfilter, this was 77% of unconjugated E1 and 82% of E2.

Acknowledgements

The Dutch Economy, Ecology, Technology (EET) programme is acknowledged for the financial support of this project.

Chapter 7

Summary and Discussion

7.1 Introduction

The first alarming messages on occurrence of hermaphrodite fish caused by the emission of compounds present in sewage treatment plant (STP) effluents came from the UK and dates back to 1993 (Ends, 1993). The suspected compounds causing this effect are: natural estrogens (steroid hormones excreted by mammals), a synthetic estrogen (excreted by women taking the contraceptive pill), and detergent breakdown products (Purdom *et al.,* 1994). Pioneers in this specific field, viz. Tabak *et al.*, already published an article on the subject in 1970, entitled "Steroid hormones as waterpollutants". A second paper followed in 1981 (Tabak *et al.,* 1981). Years later, in 1992, a subject related article of Shore *et al.* (1992) was published, dealing with the effect of two natural estrogens on crops irrigated with STP effluent.

In 1996 the US environmental protection agency recognized the adverse health effect of estrogens, referred to as endocrine disruption, as an environmental problem (Barlow, 2001). A lot of research was initiated and many scientific publications appeared since 1995. As a result, a lot of chemicals were identified to exhibit an endocrine disrupting effect. These (man-made) chemicals, called xeno-estrogens, include alkylphenols, pesticides, plasticizers (e.g. bisphenol A, phtalates), polychlorinated biphenyls, and brominated flame retardants (IEH, 1995).

In surface waters and STP effluents, xeno-estrogens are present in the micro-gram per litre range, whilst 1000-fold lower concentrations of natural estrogens estrone (E1) and 17β-estradiol (E2) and the synthetic 17α-ethynylestradiol (EE2) were detected (Chapter 2). In surface waters in the Netherlands, E1 occurs in the range of <0.3 to 7.2 ng/l, E2 in <0.3 to 1.0 ng/l and EE2 in <0.3 to 0.5 ng/l (Vethaak *et al.*, 2002). Values detected in STP effluents range from concentrations below the detection limits of the used method up to maximum values of 76 ng/l for E1 (Desbrow *et al.*, 1998) and up to 64 ng/l and 42 ng/l for E2 and EE2 respectively (Ternes *et al.*, 1999b). Johnson *et al.* (2006) calculated that the contribution of animal manure to the total emission of estrogens to surface waters is minor, viz. 15%.

Even though xeno-estrogens were found at much higher concentrations, they only contribute for 1-4% of the total estrogenic character of STP effluents (Körner *et al.*, 2000), as their estrogenic potency is relatively low. In STP effluents, over 90% of the estrogenic effect was found to be caused by E1 and E2, both natural estrogens and, the synthetic EE2 (Desbrow *et al.*, 1998; Körner *et al.*, 2001; Onda *et al.*, 2003). These compounds are biologically active at very low (environmental) concentrations; 1 ng E2/l already causes the production of the egg yolk precursor (vitellogenin) in male rainbow trout, normally only produced in female fish (Hansen *et al.*, 1998; Thorpe *et al.*, 2003). The same effect was found in rainbow trout for E1 at a concentration of 3,3 ng/l (Thorpe *et al.*, 2003). According to Metcalfe *et al.* (2001) EE2 already causes the production of oocytes in testicular tissue (ova-testis) in Medaka at a concentration of 0,03 ng/l. These values are often exceeded in environmental samples, indicating the existence of a clear potential risk to aquatic life.

Other observed effects on fish caused by the exposure to E1, E2 and EE2 comprise: fibrosis of testicular tissue (Balch *et al.*, 2004), deviated sex ratio's (Seki *et al.*, 2005), decreased reproductive success (Pawlowski *et al.*, 2004), decreased egg production (Jobling *et al.*, 2003) and decreased length (Länge *et al.*, 2001). The production of vitellogenin can also cause the loss of the male secondary sex characteristics (Hemming *et al.*, 2001). According to a number of researchers, EE2 showed the highest estrogenic potency in *in vitro* and *in vivo* tests, followed by E2 and E1 (Larsson *et al.*, 1999; Legler, 2001; Folmar *et al.*, 2002; Thorpe *et al.,* 2003).

The strong endocrine disrupting effects of E1, E2 and EE2 on aquatic life and the considerable release of these compounds to surface waters via STP effluents (Snyder *et al.*, 2001; Nakada *et al.*, 2004) and sewage overflows gave rise to research on the fate of E1, E2 and EE2 in biological treatment systems, with a focus on source separated sanitation (DeSaR) concepts.

It is important to understand that E1, E2 and EE2 enter domestic sewage via human urine and faeces. In conventional modern sanitation concepts urine and faeces are collected together with other wastewater streams from the household activities. By far the largest part of the estrogens is excreted via urine in a glucuronide or sulphate conjugated -more soluble - form in which they do not exhibit estrogenic properties (Williams and Stancel, 1996). Only a very small fraction is excreted in unconjugated form via faeces. Eighty percent of the administered dose of EE2 is excreted, of which a relatively high amount of 30% in unconjugated form via the faeces and 70% in conjugated form via the urine (Reed *et al.,* 1972). In urine over 30% is present as sulphate conjugate (Back *et al.*, 1979, 1982).

Bacterial enzymes, like β-glucuronidase, e.g. produced by *E.coli*, are capable of cleaving conjugated estrogens back to their original active form (Leggler, 2001). This deconjugation of glucuronide conjugates is likely to already proceed in the sewer (Ternes *et al.*, 1999a), whereas the deconjugation of sulphate conjugates only occurs in STPs, because it requires the presence of more specialised micro-organisms, able to produce the enzyme arylsulphatase. In raw - non inoculated - domestic wastewater, D'Ascenzo *et al.* (2003) found a significantly slower deconjugation rate of sulphate conjugates, viz. with a half-life of 3 days compared to 7 hours for glucuronide conjugates under conditions of continuous agitation.

A **source separated sanitation** concept, based on collection, transport and treatment of black water (toilet) separate from grey (shower, bath, kitchen and laundry) water, enables the recovery of energy and nutrients, and keeps estrogens in a relatively small volume (Larsen *et al.*, 2004; Chapter 1). Moreover contamination of surface waters with raw sewage due to storm water overflow is prevented. The potentials of source separated black water treatment systems, treating black water collected with vacuum toilets, is presently demonstrated in a project consisting of 32 houses in Sneek, The Netherlands[1].

The core treatment for the concentrated black water stream is anaerobic digestion. The knowledge on the fate of estrogens in anaerobic treatment systems, especially at the start of the present investigations, was extremely scarce. As post-treatment involves (micro-)aerobic systems, research has also been afforded to the fate of estrogens exposed to these conditions

[1] http://themas.stowa.nl/Themas/New_sanitation.aspx

in present research. Therefore, the obtained knowledge will also be of use for conventional STPs.

7.2 Analytical determination

The fate of estrogens can only be investigated with an accurate analytical procedure for their determination in both the solid (sludge) and liquid phase. For the detection of estrogens no standardized methods were available. Due to their very low environmental concentrations, various procedural steps are required for their accurate determination; viz.: sample pre-treatment, in order to enable the determination of the target compounds in both the liquid and the solid phase, extraction of the compounds from the solid phase, enrichment by solid phase extraction (SPE) followed by a clean up step. In the current research the reconstituted sample was detected using High Performance Liquid Chromatography (HPLC) with UV-(visible) detector (UV-VIS) for estrogen detection in the concentration range of 0.08 to 20 mg/l, Diode Array Detector (DAD) for a range of 0.02 to 10 mg/l or Fluorescence (FLU) detector in the range from 3 μg/l to 5 mg/l. Environmental relevant concentrations were, after an additional derivatisation step for increasing the volatility of the compounds, measured by Gas Chromatography (Tandem) Mass spectrometry (GC-MS(-MS)), in a range of 12 ng/l to 50 μg/l.

With new techniques, like Liquid Chromatography (Tandem) Mass spectrometry (LC-MS(-MS)) and HPLC with time of flight (TOF), a derivatisation step is no longer required. Besides these techniques enable, contrary to methods applied in current research, the detection of estrogens in conjugated form. Methods used in the present research necessitated a hydrolysis step in case information on the amounts of conjugated and unconjugated estrogens was desired. In this way it was impossible to distinguish between the amounts present as glucuronide or as sulphate conjugates.

7.3 Fate of E1, E2 and EE2 in biological treatment systems

Regarding their moderate hydrophobicity (octanol water partition coefficients around 4) adsorption of non-conjugated E1, E2 and EE2 to sludge was expected to play an important role in the removal of estrogens from the liquid phase in biological treatment systems. No significant photolytic degradation expected to contribute to their removal in STPs (Jürgens *et al.*, 1999), neither was volatilisation due to their low Henry's law constant and low vapour pressure (Rogers *et al.*, 1996).

7'.3.1 Adsorption

The values of the Freundlich adsorption coefficients (K_f) and adsorption constants (n_f) for E1, E2 and EE2 to activated sludge assessed in the current research are presented in Table 7.1 (Chapter 4 and 5). These results indeed indicate that adsorption of E1, E2 and EE2 to sludge plays an important role in the removal of the estrogens from domestic wastewater.

Table 7.1. Assessed values for the Freundlich adsorption coefficient (K_f) and constant (n_f) of E1, E2 and EE2 to activated sludge (Chapter 4 and 5).

Compound	K_f ($l^n.mg^{1-n}.kg^{-1}$)	standard deviation	n_f (-)	standard deviation
E1	100	74	4,1	0,9
E2	126	13	0,2	0,03
EE2	567	86	1,4	0,1

Because of their high adsorption coefficients (Table 7.1), underestimation of the concentrations can be induced by filtration (filter pore size of 1,2 μm) of raw domestic wastewater samples, often applied prior to further sample processing during the determination of E1, E2 and EE2 (Ternes *et al.,* 1999b; Johnson *et al.,* 2000; Laganà *et al.,* 2000; Andersen *et al.,* 2003; D'Ascenzo *et al.,* 2003). The underestimation is more profound in influents compared to effluents, as more suspended solids are present in influents.

7'.3.1 Biodegradation

Other than adsorption, biodegradation was clearly demonstrated to be an important mechanism in the removal of estrogens from domestic wastewater (Chapter 2, 4 and 5), although so far, the degradation of E1, E2 and EE2 was mainly researched under aerobic conditions with activated sludge (Chapter 2). Besides the biodegradation of E1, E2 and EE2 under aerobic conditions with activated sludge, this study also investigated biodegradation under anaerobic, anoxic, micro-aerobic and nitrifying inhibited conditions with various types of sludge.

The assessed mean specific first order degradation constants (specific *k*-values) in the biodegradation experiments with activated sludge under **aerobic** conditions are presented in Table 7.2. Under these conditions E2 is rapidly converted into E1, and then E1 is mineralised. EE2 was clearly found most persistent, with the lowest assessed *k*-value. Membrane bioreactor (MBR) sludge with long sludge retention times (SRT), was reported to degrade all three compounds at a higher rate than activated sludge (Joss *et al.*, 2004). This was confirmed in present research as MBR sludge obtained the highest specific *k*-value for the degradation of EE2 under aerobic conditions (Table 7.2).

Aerobic degradation rates assessed in present research at relatively high spiking concentrations of 5 mg/l, were significantly lower compared to those calculated from results of experiments conducted at low initial estrogen concentrations in the ng/l and μg/l range (Chapter 2, 4 and 5, Table 7.2), particularly for E1 and EE2. Several researches indicated toxicity to be unlikely at the applied concentrations (5 mg/l), since no inhibition of nitrifying sludge was manifested at E2 concentrations up to 60 mg/l (Kozak *et al.,* 2001), whilst pure cultures spiked with up to 100 mg E2/l and 20 mg EE2/l showed a rapid estrogen degradation (Fujii *et al.*, 2002; Yoshimoto *et al.,* 2004; Scheweinfurth *et al.,* 1996). Obviously, other processes than biodegradation itself are rate limiting, and manifest at high spiking concentrations. Results from the present research indicate a decreased bioavailability, i.e. desorption, as the rate limiting step during degradation of estrogens by activated sludge.

Results from a desorption experiment, executed with EE2 addition to activated sludge, show the presence of a rapidly desorbing fraction of 0.58, whilst of the remaining EE2 a fraction of 0.34 was not released from the sludge (Chapter 5). The occurrence of desorption appears likely; when a distinction was made between a rapid and a slow degradable fraction (two compartmental approach) a huge similarity was found between the rapidly degrading fraction in the aerobic biodegradation of EE2 (F_{rapid}= 0.51) and the rapidly desorbing fraction (Chapter 5).

Another indication of rate limitation due to desorption can be found in the calculated adsorption coefficients (K_f values) for the adsorption of E1 and EE2, because they were always higher in the aerobic biodegradation experiments than those found in the short-term adsorption experiments, indicating that a relative high amount is sorbed during the degradation experiments. Also the higher specific degradation constants for E1 found in experiments with diluted sludge, which implies that less sludge is available for adsorption, reveal an increased bioavailability, since a significant larger fraction was degraded at the rapid first order rate (Chapter 4).

Table 7.2. Specific first order degradation constants (specific *k*-values) as found in present research under aerobic conditions in comparison with literature values.

Compound	Sludge type	specific *k*-value (l.g $TSS^{-1}.d^{-1}$)	Temperature (°C)	Reference
E1	Activated sludge	0.27	23	Chapter 4
E1		162	16	Joss *et al.* (2004)
E2		97	23	Chapter 4
E2		350	16	Joss *et al.* (2004)
EE2		0.029	19-24	Chapter 5
EE2		8	16	Joss *et al.* (2004)
EE2	MBR sludge	0.06	17-25	Chapter 5
EE2		6	16	Joss *et al.* (2004)
EE2	SBR sludge	0.094	19	Chapter 5

Results of batch experiments conducted with activated sludge under aerobic conditions and **nitrification inhibition,** did not show significantly lower *k*-values for E1 and EE2 degradation when compared to those found under aerobic conditions with nitrification (Chapter 4 and 5). Pure cultures of nitrifying bacteria have shown to be capable to degrade EE2 (Shi et al., 2004a/b). Yi and Harper (2007) found a linear relation between the rate of ammonia oxidation and EE2 degradation in a pure culture of autotrophic ammonia oxidizers and they concluded that the biotransformation proceeded co-metabolically mediated in the presence of ammonium monooxygenase (AMO). As found in the present study not only nitrifiers are capable to convert EE2; in experiments conducted with sludge from a sequencing batch reactor (SBR), lacking nitrifying capacity, biodegradation of EE2 manifested under aerobic conditions. In these latter experiments a high specific *k*-value for the degradation of EE2, viz. 0.094 l.g $TSS^{-1}.d^{-1}$ (Chapter 5) was obtained, which is in contrast with results of

Ternes *et al.* (1999a) and Vader *et al.* (2000), who reported absence of any degradation of EE2 when using activated sludge without nitrifying capacity.

So far, no information was available in literature on the degradation of E1, E2 and EE2 under oxygen limited, viz. **micro-aerobic**, conditions (O_2 concentration < 1 mg/l). The results obtained in the present investigations reveal a distinctly lower degradation rate when compared to aerobic conditions; the specific *k*-values found amounted to 0.10 and 0.006 l.g $TSS^{-1}.d^{-1}$ for E1 and EE2 (Chapter 4 and 5).

Under **anoxic** conditions the conversion of E1 and E2 proceeds much slower compared to aerobic conditions; the assessed conversion rates for EE2 were close to the rates observed in the abiotic controls. Anoxic conditions gave a low E1 degradation rate of approximately 0.004 l.g $TSS^{-1}.d^{-1}$ and no degradation was observed for EE2, neither after substrate addition and respiking. Fahrbach *et al.* (2006) isolated a gram negative denitrifying bacterium from activated sludge which was capable of completely mineralising E2, but information on its ability to degrade EE2 is lacking.

Results of **anaerobic** short-term biodegradation experiments, conducted with activated sludge, showed no decline in the sum of E1 and E2 concentrations; the dominant process in these experiments appeared to be the conversion of E1 into E2 (Chapter 4), although some re-conversion of E2 into E1 manifested in these experiments. Under similar conditions, no decline in the concentration of EE2 was observed, neither after addition of substrate or respiking (Chapter 5).

Results of long term (205-256 d) anaerobic biodegradation tests conducted with different types of sludge, viz. including digested pig manure, granular UASB sludge, UASB septic tank sludge and activated sludge likewise did not reveal any decline in the sum of E1 and E2. The rate and extent of the conversion of E1 into E2 was found to vary for the different types of sludges (Chapter 4). In similar long term experiments no decline in the concentration of EE2 manifested (Chapter 5), which is in agreement with findings of Czajka and Londry (2006) who found no EE2 degradation in spiked sediments and surface water batch experiments under anaerobic conditions over a period of 3 years, not even in presence of the oxidants NO_3, SO_4^{2-} or Fe^{3+}. This is in contrast with findings of Carballa *et al.* (2006) who reported a removal of 85±5% and 75±15% for EE2 in continuous sludge digestion experiments under respectively mesophilic and thermophilic conditions, although this only occurred after sludge adaptation (duration not reported). Regarding the different observation in the present research, it is clear that this remains to be elucidated in future. Interestingly, activated sludge samples kept under anaerobic conditions for 200 days, are still capable of degrading EE2 once oxygen is supplied into the test bottle (Chapter 5); this might indicate facultative aerobic bacteria are responsible for the degradation.

7.4 Fate of estrogens in conventional STPs

Biodegradation rates and adsorption constants for estrogens established in batch experiments can be used to calculate their effluent concentrations in activated sludge systems treating domestic sewage. In the present research, effluent estrogen concentrations were averaged from reported values in literature, as measured in full-scale STPs (Chapter 2); they amounted

to 13.9±14.3 ng/l for E1, 2.1±2.7 ng/l for E2 and 1.7±4.3 ng/l for EE2. The calculated average removal percentages were 62±27%, 88±9% and 56±24% for E1, E2 and EE2 respectively. Systems with nitrification and denitrification showed optimum removal of E1, E2 and EE2.

Adsorption and biodegradation of estrogens in an activated sludge system can be modelled using the method proposed by Temmink, (2001), viz. Equation 1. The activated sludge system is assumed to behave as a continuous, completely mixed reactor. Process parameters are chosen based on average values for conventional activated sludge systems with nitrification/denitrification.

$$C_e = \frac{Q_i \cdot C_i}{\frac{1 + K_d \cdot X_a}{1 + K_d \cdot X_e} \cdot (Q_w + V_a \cdot k \cdot X_a) + Q_i - Q_w} \qquad \text{Equation 1}$$

In which:

C_e: Concentration of target compounds in effluent of activated sludge treatment (ng/l)

C_i: Averaged concentration of target compounds in influents of activated sludge treatment (ng/l)

X_a: Biomass concentration in activated sludge system; value: 4 g TSS/l, mostly applied in conventional STPs

X_e: Biomass concentration in the effluent; set on 0.02 g/l (assumption)

Q_i: Flow of the liquid phase; assuming 10000 inhabitant equivalents (1 inhabitant equivalent= 136 g COD.person^{-1}.d^{-1}), dry-weather flows: 1300 m^3/d, for wet-weather flows: 2000 m^3/d.

Q_w: Flow of the waste sludge; Calculation based on a sludge retention time (SRT) of 12 d, which is a common value for an activated sludge system employing nitrification/denitrification (Andersen *et al.*, 2003)

V_a: Volume aeration tank (m^3); calculated with common sludge load of 0.25 kg COD.kg TSS^{-1}.d^{-1} (Metcalf and Eddy, 2003); 1360 m^3

k: Specific first order degradation constant, Table 7.2 (l.g TSS^{-1}.h^{-1})

K_d: Partition coefficient solid-water in suspended matter; E1: 0.402 l/g TSS, E2: 0.476 l/g TSS, EE2: 0.584 l/g (Andersen *et al.,* 2005)

Results of the model calculations are presented in Figure 7.1. Both the relatively low specific first order degradation constant as obtained in present research and the high values achieved by Joss *et al.* (2004) were used in the calculation. The averaged measured effluent values for full scale treatment systems for domestic sewage (Chapter 2) are also depicted in Figure 7.1. Apparently the averaged measured effluent concentrations are better predicted when using the low specific first order degradation values (*k*-values) as assessed in the present research. Measured higher effluent estrogen concentrations may be attributed to the fact that STPs are not completely mixed systems in practice. As a result, zones with an oxygen deficiency and short-circuiting/channelling may prevail in full scale installations and these reduce the actual hydraulic retention time. Besides, conversion rate limitation due to decreased bioavailablity as a result of e.g. desorption will also have its share. Moreover, influent estrogen concentrations might have been underestimated due to the unmeasured fraction associated with particles and

to insufficient data on the fraction of conjugated estrogens in STP influents, their role may be greater than anticipated.

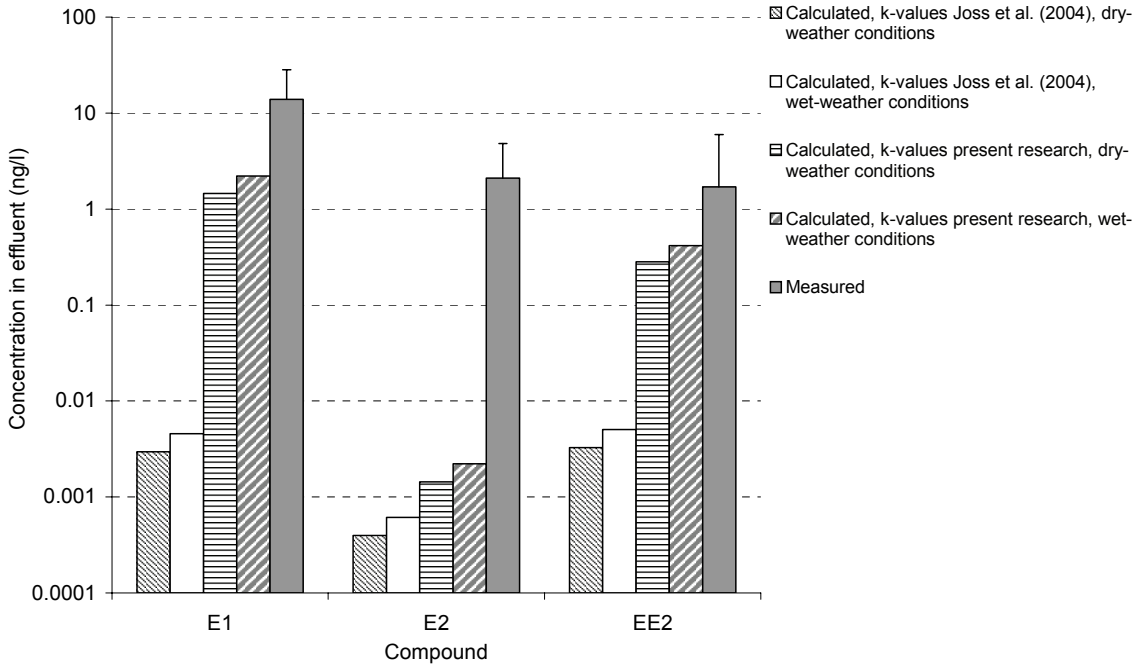


Figure 7.1. Modelled and average measured values for concentrations of E1, E2 and EE2 in STP effluents (logarithmic scale), assessed with average measured influent concentrations and first order degradation constants (*k*-values) from Joss *et al.* (2004) performed with low initial estrogen spiking concentrations and k-values from current research (Equation 1). Measured values are averaged final effluent values (Chapter 2).

7.5 Fate of estrogens in innovative black water treatment systems

7.5.1 Anaerobic pre-treatment

The results presented in Chapter 6 show the presence of substantial concentrations of E1 and E2 in the effluent of the pilot scale UASB septic tank-system treating black water (collected using vacuum toilets), viz. 4.02±0.52 µg/l and 18.69±10.04 µg/l for E1 and E2 respectively. These values comprise the sum of the conjugated and unconjugated compounds. The higher concentration measured for E2 is in compliance with the finding that E1 is reduced to E2 under anaerobic conditions (Chapter 4). A large percentage of the natural estrogens was present in the UASB effluent in the conjugated form, viz. >70% for E1 and >80% for E2. 53% of unconjugated E1 and 25% of unconjugated E2 was associated with particles >1.2 µm. The higher adsorption of E1 to particles than E2 conformed with the assessed K_f adsorption values (Chapter 4). No EE2 could be detected by GC-MS in the UASB-septic tank effluent.

Whether deconjugation of the 20-30% natural estrogens occurred during anaerobic treatment or in the transport/storage line is unknown. According to D'Ascenzo *et al.* (2003) a small amount of glucuronide conjugates was deconjugated during storage in a septic tank, while sulphate conjugates remained unchanged.

Based on results of anaerobic degradation tests of natural and synthetic estrogens (Chapter 4 and 5), no degradation is expected to occur in the UASB-septic tank. As estrogens are shown to be present mainly in the conjugated form, which is soluble in water, also adsorption might be very limited, which then will result in a restricted removal in anaerobic processes. With an expected total concentration of 42 µg/l in the UASB septic tank influent (De Mes and Zeeman, 2003) at a flush volume of 0.5 L, the maximum removal of both E1 and E2 comprise to only 46%.

7.5.2 Post-treatment

In the final effluent of the post-treatment systems, viz. consisting of two down hanging sponge (DHS) reactors in series followed by a sand filter, E1 and E2 were still present in substantial concentrations, viz. a total concentration of 1.37±1.45 µg/l and 0.65±0.78 µg/l, respectively. Percentages of 77% and 82% of the measured unconjugated E1 and E2 were associated with particles (>1.2 µm) in the final effluent (Chapter 6).

When spiking the influent of the post-treatment with E1, E2, EE2 and the sulphate conjugate of E2, removal was >99% for both E2 and EE2 and 83% for E1; 40% of E1 and 99% of E2 was present in conjugated form in the final effluent. As no conjugated E1 was added, this value is completely due to the amount naturally present in black water. In general, conjugated estrogens are not determined during monitoring of full scale STPs (Chapter 2). A remarkable amount is still present in conjugated form after anaerobic treatment (70 and 80% for E1 and E2) and micro-aerobic post-treatment (40% of E1). This concurs with observations of Adler (2001) and Gentili *et al.* (2002) showing that conjugated estrogens still contributed significantly (over 40%) to the total estrogen concentrations in the effluents of STPs. Both the results of spiked and non spiked experiments show that deconjugation is not occurring as fast as generally assumed.

Regarding the presence of the substantial amounts of E1 and E2 in the final effluent of the post-treatment, the application of an additional treatment, like an ozonation step, is inevitable. According to Escher *et al.* (2006) ozonation of urine results in a high removal of estrogens (>99%) besides ozonation is effective for a wide range of pharmaceuticals (Snyder *et al.*, 2005). Other effective advanced post-treatment systems for the removal of E1, E2 and EE2 might be nanofiltration and utlrafiltration (Yoon *et al.*, 2006), reverse osmosis and electrodialyses (Maurer *et al.*, 2006) and UV-radiation (Snyder *et al.*, 2005). Also a membrane bioreactor as tertiary treatment, which can also serve as an alternative to conventional activated sludge systems can be mentioned. MBR sludge showed higher degradation rates for estrogens. However in practice, final effluent values were not necessarily lower (Hu *et al.,* 2007; Clara *et al.*, 2005). This might be attributed to the lower overall HRT in an MBR, although higher sludge concentrations can be applied, so the sludge load stays constant. Urmenyi *et al.* (2005) showed a good possibility to remove specific estrogens with a membrane containing build-in antigens. However, at the current stage this option is too expensive in this particular application.

The advantage of a source separated sanitation concept over a conventional sanitation concept is that nearly all estrogens are present in a small volume, viz. the black water stream

which is as well the case for many other pharmaceuticals excreted by humans. Regarding the extremely small volume, viz. 7 $l.p^{-1}.d^{-1}$, in comparison to water volume in conventional domestic wastewater collection/treatment concepts,
viz. ≥ 130 $l.p^{-1}.d^{-1}$, the required supplementary treatment can remain relatively compact.

7.6 Concluding remarks and future research perspectives

As in previously performed researches, the synthetic estrogen 17α-ethynylestradiol can be designated as the most persistent of the three compounds investigated in the present research. Although an increasing sludge retention time clearly exerts a positive effect on the removal rates prevailing in activated and membrane bioreactor sludge, the results obtained in the present investigations reveal that the ability of the sludge for nitrification clearly is not a prerequisite.

For the degradation of all three estrogens, aerobic redox conditions are most favourable. The results of batch experiments performed under laboratory controlled conditions can not be directly translated to the performance of full scale installations. In practice, other processes than biodegradation influence the removal efficiency. Besides, present research employed high initial concentrations of estrogens (over 100 times for E1 and E2 and over 1000 times for EE2), which will never occur in domestic wastewaters. However, it was suitable to compare the different applied conditions and to determine possible rate limiting processes. The present research clearly indicates that desorption of adsorbed estrogens can result in a lower first order degradation constant, and therefore a lower overall removal.

The data collected in present research could form an input for modelling in order to further develop the interrelations between the different parameters concerning the fate in full scale treatment systems, eventually supported by some additional experiments.

Moreover, an underestimation of the concentration of estrogens present in influents can contribute to effluent concentrations higher than expected on base of degradation experiments. Underestimation can be due to the fact that estrogens associated with particles are not taken into account and due to the lack of information on the contribution of estrogens still present in conjugated form. Besides, no information is available on the fate of the conjugates under different redox conditions. Results presented in this thesis indicate only limited deconjugation of the natural estrogens estrone and 17β-estradiol during anaerobic treatment. There is clearly a need to elucidate their degree of deconjugation as well as their binding properties.

Even though STP effluents are considered to play a major role in the contribution of endocrine disrupters to surface waters, the role of manure application in agricultural areas should not be completely ignored. Locally, high concentrations in drainage waters near manure treated-fields were observed (Kjær *et al.*, 2007).

The latest developments in the field of DeSaR in the Netherlands are known under the name "new sanitation", and involves the separate collection of urine (www.stowa.nl). This offers a very good solution for pollution control in terms of nutrients (Wilsenach *et al.*, 2005; Wilsenach, 2006). Care must be taken to apply this as a solution for the prevention of

estrogens and pharmaceuticals entering the environment. On average still 30% of pharmaceuticals are excreted with faeces (Alder *et al.*, 2006) and the environmental risk potential was estimated to be almost equal in urine and faeces (Lienert *et al.*, 2006). Lienert *et al.* (2007) hypothesized that as pharmaceuticals excreted in faeces are generally more hydrophobic and have a tendency to sorb to sludge and be therefore better removable than the hydrophilic substances in urine. In case of estrogens, combining urine and faeces enhances the appearance of an increased amount of more hydrophilic compounds by deconjugation. However, further investigations are necessary to be able to draw clear conclusions.

The incomplete removal of estrogens in biological treatment systems necessitates the application of supplementary treatment. Even though the removal of estrogens from biological treatment systems of concentrated black water will be poor, the source separation based sanitation concept is still highly preferable over conventional concepts, regarding the fact that all the estrogens, like a variety of pharmaceuticals are present in a very small volume. This offers good opportunities for the application of compact and efficient tertiary treatment systems, following the anaerobic and (micro-)aerobic treatment steps. Source separated sanitation systems completely eliminate the risk of surface water contamination by storm-water overflows.

Hoofdstuk 7'

Samenvatting en Discussie

7'.1 Inleiding

De eerste alarmerende berichten over het voorkomen van hermafrodiete vissen, veroorzaakt door de emissie van componenten aanwezig in effluenten van rioolwaterzuiveringsinstallaties (RWZI's) dateren van 1993 (Ends, 1993). De verdachte componenten verantwoordelijk voor dit effect waren: natuurlijke oestrogenen (steroïde hormonen, uitgescheiden door zoogdieren), een synthetisch oestrogeeen (uitgescheiden door vrouwen aan de anticonceptie pil) en afbraakproducten van detergenten (Purdom *et al.,* 1994). Pioniers op dit specifieke onderwerp, te weten Tabak *et al.*, publiseerden al een artikel op dit onderwerp in 1970 met de titel "Steroid hormones as waterpollutants". Een tweede artikel volgde in 1981 (Tabak *et al.,* 1981). Pas jaren later, in 1992, verscheen er een onderwerp gerelateerd artikel door Shore *et al.* (1992), welke handelde over het effect van twee natuurlijke oestrogenen op gewassen door irrigatie met RWZI effluent.

In 1996 werd de negatieve gezondheidsimpact van oestrogenen erkend als milieuprobleem door het "Environmental protection agency" (EPA) in de VS, en aangemerkt als hormoonverstoring (Barlow, 2001). Dit leidde tot de aanzet van veel wetenschappelijk onderzoek en vanaf 1995 zijn er vele artikelen verschenen. Dit resulteerde in de identificatie van een scala aan chemicaliën welke een hormoonverstorend effect bleken te bezitten. Deze (synthetische) chemicaliën, xeno-oestrogenen genaamd, omvatten alkylfenolen, pesticiden, weekmakers (zoals bisfenol A, ftalaten), gechloreerde bifenylen en gebromineerde brandvertragers (IEH, 1995).

In oppervlaktewateren en RWZI effluenten komen xeno-oestrogenen voor in een concentratie van microgrammen per liter, terwijl de natuurlijke oestrogenen, oestron (E1) en 17β-oestradiol (E2), en het synthetische 17α-ethynyloestradiol (EE2) in duizend maal lagere concentraties zijn gedetecteerd (Hoofdstuk 2). In oppervlaktewateren in Nederland komt E1 voor in concentraties van <0.3 tot 7.2 ng/l, E2 van <0.3 tot 1.0 ng/l en EE2 van <0.3 tot 0.5 ng/l (Vethaak *et al.*, 2002). Gedetecteerde waarden in RWZI effluenten liggen van beneden de detectie limiet tot een maximum waarde van 76 ng/l voor E1 (Desbrow *et al.*, 1998), tot 64 ng/l en 42 ng/l voor respectievelijk E2 en EE2 (Ternes *et al.*, 1999b). Johnson *et al.* (2006) heeft berekend dat van de totale emissie van oestrogenen naar oppervlaktewateren, het aandeel vanuit dierlijke mest slechts 15% bijdraagt.

Ondanks het feit dat xeno-oestrogenen in een veel hogere concentratie voorkomen, dragen zij slechts voor 1-4% bij aan het oestrogene karakter van RWZI effluenten (Körner *et al.*, 2000), wat wordt veroorzaakt door hun relatief lage oestrogene potentie. Meer dan 90% van het hormoonverstorende effect in RWZI effluenten kan toegeschreven worden aan E1 en E2, beide natuurlijke oestrogenen, en het synthetische EE2 (Desbrow *et al.*, 1998; Körner *et al.*, 2001; Onda *et al.*, 2003). Deze componenten zijn in zeer lage (milieu) concentraties biologisch actief; zo veroorzaakt 1 ng E2/l al de productie van een dooiereiwit (vitellogenine) in mannetjes regenboogforellen, welke normaal slechts geproduceerd wordt in vrouwelijke vissen (Hansen *et al.*, 1998; Thorpe *et al.*, 2003). Hetzelfde effect is gevonden voor E1 in een concentratie van 3.3 ng/l (Thorpe *et al.*, 2003). Metcalfe *et al.* (2001) liet zien dat een blootstelling aan 0.03 ng/l EE2 al productie van eicellen in testikel weefsel (ova-testis) in

medaka (Japans rijstvisje) veroorzaakt. Deze waarden worden vaak overschreden in oppervlaktewatermonsters, waardoor er een potentieel risico voor het watermilieu is.

Andere waargenomen effecten op vissen, veroorzaakt door de blootstelling aan E1, E2 en EE2 zijn: fibrose van testikel weefsel (Balch *et al.*, 2004), een verschoven sekse verdeling (Seki *et al.*, 2005), verminderde reproductie successen (Pawlowski *et al.*, 2004), verminderde ei productie (Jobling *et al.*, 2003) en verminderde lengte (Länge *et al.*, 2001). De productie van vitellogenine kan ook leiden tot het verlies van de mannelijke secundaire geslachtskenmerken (Hemming *et al.*, 2001). Een aantal onderzoekers laat zien dat EE2 in *in vitro* en in *in vivo* testen de hoogste oestrogene potentie heeft, gevolgd door E2 en E1 (Larsson *et al.*, 1999; Legler, 2001; Folmar *et al.*, 2002; Thorpe *et al.,* 2003).

Het sterke hormoonverstorende effect van E1, E2 en EE2 op het watermilieu en de uitstoot van deze componenten in oppervlaktewater via RWZI effluenten (Snyder *et al.*, 2001; Nakada *et al.*, 2004) en riooloverstorten, hebben ertoe geleid dat dit onderzoek naar het gedrag van E1, E2 en EE2 in biologische behandelingssystemen, met de nadruk op brongescheiden sanitatie (DeSaR) concepten, is uitgevoerd.

Een belangrijk gegeven is dat de oorsprong van E1, E2 and EE2 in huishoudelijk afvalwater, menselijke urine en feces is. In moderne conventionele sanitatie concepten worden urine en feces gezamenlijk met de andere afvalwaterstromen van het huishouden ingezameld. Het grootste deel van de oestrogenen wordt uitgescheiden in de urine als in water goed oplosbare glururonide- of sulfaatconjugaten, welke op zich geen oestrogene activiteit bezitten (Williams and Stancel, 1996). Slechts een klein deel wordt uitgescheiden met de feces, in ongeconjugeerde vorm. Tachtig procent van de ingenomen hoeveelheid EE2 wordt uitgescheiden, waarvan een relatief groot deel in ongeconjugeerde vorm in de feces en 70% als conjugaat in de urine (Reed *et al.,* 1972). In de urine is meer dan 30% aanwezig als sulfaatconjugaat (Back *et al.*, 1979, 1982). Bacteriologische enzymen, zoals β-glucuronidase, bijvoorbeeld geproduceerd door *E.coli*, zijn in staat om conjugaten te splitsen naar hun originele actieve vorm (Leggler, 2001). Voor glucuronideconjugaten wordt deze deconjugatie al verondersteld plaats te vinden in het riool (Ternes *et al.*, 1999a), terwijl dit voor sulfaatconjugaten pas plaatsvindt in RWZI's, omdat voor de productie van sulfatase meer gespecialiseerde micro-organismen nodig zijn. D'Ascenzo *et al.* (2003) vonden in ruw – niet geënt – huishoudelijk afvalwater, een significant lagere deconjugatie snelheid voor sulfaatconjugaten, met een halfwaarde tijd van 3 dagen, tegen 7 uur voor glucuronideconjugaten onder voortdurend roeren.

Een **brongescheiden sanitatie** concept is gebaseerd op gescheiden inzameling, transport en behandeling van zwartwater (toilet), grijswater (douche, bad, keuken en was), waardoor het terugwinnen van energie en nutriënten mogelijk is, waardoor tevens de oestrogenen aanwezig zijn in een veel kleiner volume (Larsen *et al.*, 2004; Hoofdstuk 1). Tevens wordt de contaminatie van oppervlaktewateren met ruw afvalwater als gevolg van overstorten, voorkomen. De potentie van zwartwater behandelingssystemen wordt momenteel gedemonstreerd in Sneek voor 32 huishoudens[1].

Anaërobe vergisting is de kern techniek voor de behandeling van zwartwater. De kennis over het gedrag van oestrogenen in anaërobe behandelingssystemen was, zeker bij

[1] http://themas.stowa.nl/Themas/New_sanitation.aspx

aanvang van dit onderzoek, zeer schaars. De nabehandeling bestaat uit (micro-)aërobe systemen, zodat de resultaten van dit onderzoek ook toepasbaar zijn voor conventionele zuiveringen.

7'.2 Analytische bepalingen

Het gedrag van oestrogenen kan alleen onderzocht worden met behulp van accurate analytische procedures voor de bepaling in zowel de vaste (slib) als de vloeibare fase. Voor de detectie van oestrogenen in water en slib zijn geen gestandaardiseerde methoden beschikbaar. Door de lage concentratie in milieucompartimenten zijn verscheidene stappen noodzakelijk om de concentratie accuraat te kunnen bepalen, te weten: monster voorbehandeling, om de slib en water fase te scheiden voor afzonderlijke bepaling, extractie van de componenten van de vaste fase, verrijking door "vaste fase extractie" (solid phase extraction (SPE)), gevolgd door een opschoningstap. Gedurende dit onderzoek was een gereconstitueerd monster gemeten op een HPLC (vloeistof chromatografie) met een UV-detector voor de detectie van oestrogenen in het concentratie bereik van 0.08 tot 20 mg/l, met een DAD (Diode Array Detector) voor een bereik van 0.02 tot 10 mg/l of een fluorescentie (FLU) detector voor een bereik van 3 µg/l tot 5 mg/l. Milieurelevante concentraties, zijn na een additionele derivatisatiestap om de vluchtigheid te verhogen, gemeten op een GC-MS(-MS) (Gas chromatografie met massa spectrometrie) in een concentratie bereik van 12 ng/l tot 50 µg/l.

Voor nieuwe technieken, zoals vloeistof chromatografie in combinatie met massa spectrometrie en HPLC met TOF (time of flight), is geen derivatisatiestap benodigd. Daarnaast kunnen deze technieken, in tegenstelling tot de methodieken gebruikt in dit onderzoek, oestrogenen detecteren in geconjugeerde vorm. De gebruikte technieken waren alleen in staat om conjugaten te meten door een extra hydrolyse stap toe te passen, indien er informatie over zowel geconjugeerde als ongeconjugeerde oestrogenen gewenst was. Op deze manier was het niet mogelijk om inzicht te krijgen in de verdeling tussen de hoeveelheden die geconjugeerd zijn als glucuronide- of als sulfaatconjugaat.

7'.3 Gedrag van E1, E2 en EE2 in biologische behandelingssystemen

Gezien de gematigde hydrofobiciteit (octanol water partitie coëfficiënten rond de 4) wordt adsorptie aan slib van de niet-geconjugeerde E1, E2 en EE2 verondersteld een belangrijke rol in te nemen bij de verwijdering van oestrogenen uit de vloeistoffase in biologische systemen. De bijdrage van fotolytische degradatie aan de verwijdering in RWZI's is niet significant (Jürgens *et al.*, 1999), benevens vervluchtiging gezien hun lage Henry's law constante en lage dampspanning (Rogers *et al.*, 1996).

7'.3.1 Adsorptie

De waarden van de Freundlich adsorptie coëfficiënten (K_f) en constanten (n_f) voor E1, E2 en EE2 aan actief slib zoals gevonden in dit onderzoek zijn weergegeven in Tabel 7'.1 (Hoofdstuk 4 en 5). Deze resultaten impliceren inderdaad dat adsorptie van E1, E2 en EE2

aan slib een belangrijke rol speelt in de verwijdering van oestrogenen uit huishoudelijk afvalwater.

Tabel 7'.1. Waarden voor de Freundlich adsorptie coëfficiënten (K_f) en constanten (n_f) van E1, E2 en EE2 aan actief slib (Hoofdstuk 4 en 5).

Component	K_f ($l^n.mg^{1-n}.kg^{-1}$)	Standaarddeviatie	n_f (-)	standaarddeviatie
E1	100	74	4,1	0,9
E2	126	13	0,2	0,03
EE2	567	86	1,4	0,1

Gezien de hoge adsorptie coëfficiënten (Tabel 7'.1) kan de frequent toegepaste filtratie stap (filter poriegrootte van 1,2 μm), alvorens verdere opwerking van een ruw afvalwater monster, leiden tot een onderschatting van de concentratie van E1, E2 and EE2 (Ternes *et al.,* 1999b; Johnson *et al.,* 2000; Laganà *et al.,* 2000; Andersen *et al.,* 2003; D'Ascenzo *et al.,* 2003). Deze onderschatting is relatief groter in influenten vergeleken met effluenten, daar er meer gesuspendeerd materiaal in influent aanwezig is.

7'.3.2 Biodegradatie

Naast adsorptie is aangetoond dat biodegradatie een belangrijk mechanisme is voor de verwijdering van oestrogenen uit huishoudelijk afvalwater (Hoofdstuk 2, 4 en 5).Tot op heden is vooral aandacht besteed aan de afbraak van E1, E2 en EE2 onder aërobe omstandigheden in actief slib (Hoofdstuk 2). Naast de biologische afbraak van E1, E2 en EE2 onder aërobe omstandigheden is in deze studie ook de afbraak onderzocht onder anaërobe, anoxische, microaërobe en nitrificatie geremde condities met verschillende soorten slib. De gevonden gemiddelde specifieke eerste orde afbraakconstanten (specifieke *k*-waarden) in afbraakexperimenten met actief slib onder **aërobe** omstandigheden zijn weergegeven in Tabel 7'.2. Onder deze condities wordt E2 zeer snel omgezet naar E1, waarna E1 wordt gemineraliseerd. EE2 was duidelijk het meest persistent, met de laagste *k*-waarde. Voor membraan bioreactor (MBR) slib met lange slibleeftijden zijn afbraaksnelheden gerapporteerd die voor alle drie de stoffen structureel hoger liggen dan voor actief slib (Joss *et al.*, 2004). Dit is bevestigd in dit onderzoek: met MBR slib zijn de hoogste specifieke *k*-waarden voor de afbraak van EE2 onder aërobe condities gevonden (Tabel 7'.2).

Aërobe afbraaksnelheden gevonden in dit onderzoek met een relatief hoge doseringsconcentratie van 5 mg/l, waren significant lager vergeleken met waarden gevonden in experimenten die zijn uitgevoerd bij lage initiële concentraties van ng/l en μg/l (Hoofdstuk 2, 4 en 5, Tabel 7.2), met name voor E1 en EE2. Verscheidene onderzoeken hebben aangetoond dat toxiciteit onwaarschijnlijk is bij de toegepaste concentraties (mg/l), omdat er geen inhibitie is opgetreden in nitrificerend slib blootgesteld aan E2 concentraties tot 60 mg/l (Kozak *et al.,* 2001), terwijl reinculturen gedoseerd tot concentraties van 100 mg E2/l en 20 mg EE2/l nog steeds een snelle afbraak van de oestrogenen lieten zien (Fujii *et al.*, 2002; Yoshimoto *et al.,* 2004; Scheweinfurth *et al.,* 1996). Het is duidelijk dat andere processen dan

de biodegradeerbaarheid zelf snelheidsbeperkend zijn en zich mogelijk pas manifesteren bij een wat hogere doseringen van oestrogenen. Resultaten van dit onderzoek wijzen in de richting van een verminderde biologische beschikbaarheid, waarbij mogelijk desorptie de snelheidsbepalende stap gedurende de afbraak van oestrogenen in actief slib is. Resultaten van een desorptie experiment uitgevoerd met EE2 toevoeging aan actief slib laat een snel desorberende fractie zien van 0.58, terwijl een resterende fractie van 0.34 niet van het slib is gedesorberd (Hoofdstuk 5). De beperkte desorptie lijkt aannemelijk; wanneer er onderscheid wordt gemaakt tussen een snelle en een langzame afbreekbare fractie (twee compartimenten benadering) wordt een overeenstemming gevonden tussen de snel afbreekbare fractie tijdens de aërobe biodegradatie van EE2 (F_{snel}= 0.51) en de fractie die snel desorbeert (Hoofdstuk 5). Een andere aanwijzing voor de snelheidsbeperking door desorptie is te vinden in de hogere adsorptiecoëfficiënten (K_f waarden) die zijn gevonden tijdens afbreekbaarheidexperimenten voor E1 en EE2 vergeleken met de waarden gevonden in korte-termijn adsorptie experimenten, zodat een relatief grote hoeveelheid oestrogenen gebonden is tijdens het afbreekbaarheidexperiment. Ook de hogere specifieke degradatie constante voor E1 in experimenten uitgevoerd met verdund slib, geeft een significante grotere fractie welke afgebroken werd volgens de snelle eerste orde snelheid, waarschijnlijk omdat er minder slib aanwezig is voor adsorptie (Hoofdstuk 4).

Tabel 7'.2. Specifieke eerste orde degradatie constanten (specifieke *k*-waarden) gevonden in dit onderzoek onder aërobe omstandigheden in vergelijking tot literatuur waarden.

Component	Slib type	Specifieke *k*-waarde ($l.g\ TSS^{-1}.d^{-1}$)	Temperatuur (°C)	Referentie
E1	Actief slib	0.27	23	Hoofdstuk 4
E1		162	16	Joss *et al.* (2004)
E2		97	23	Hoofdstuk 4
E2		350	16	Joss *et al.* (2004)
EE2		0.029	19-24	Hoofdstuk 5
EE2		8	16	Joss *et al.* (2004)
EE2	MBR slib	0.06	17-25	Hoofdstuk 5
EE2		6	16	Joss *et al.* (2004)
EE2	SBR slib	0.094	19	Hoofdstuk 5

Resultaten van batch experimenten uitgevoerd met actief slib onder aërobe condities en **nitrificatie inhibitie,** leidden niet tot significant lagere *k*-waarde voor E1 en EE2 degradatie vergeleken met de waarden gevonden onder aërobe condities met nitrificatie (Hoofdstuk 4 en 5). Reinculturen van nitrificerende bacteriën zijn in staat om EE2 af te breken (Shi et al., 2004a/b). Yi en Harper (2007) vonden een lineaire relatie tussen de snelheid van ammonia oxidatie en EE2 degradatie in een reincultuur bestaande uit autotrofe ammonia oxideerders en zij concludeerden dat de biotransformatie plaatsvindt volgens co-metabolisme in aanwezigheid van ammonium monooxygenase (AMO). Dit onderzoek heeft laten zien dat niet alleen de nitrificeerders in staat zijn om EE2 om te zetten; in experimenten uitgevoerd met slib uit een zogenaamde sequencing batch reactor (SBR), welke geen nitrificerende capaciteit

had, werd ook biodegradatie van EE2 gevonden onder aërobe condities. In deze experimenten werd zelfs een hoge specifieke *k*-waarde voor de afbraak van EE2 gevonden, te weten 0.094 l.g $TSS^{-1}.d^{-1}$ (Hoofdstuk 5), wat enigszins in tegenstelling staat tot resultaten van Ternes *et al.* (1999a) en Vader *et al.* (2000), die geen afbraak rapporteerden met actief slib zonder nitrificatie capaciteit.

Tot op heden was nog niet gerapporteerd in de literatuur over de afbreekbaarheid van E1, E2 en EE2 onder zuurstof limiterende, ofwel **microaërobe**, condities (O_2 concentratie < 1 mg/l). De resultaten verkregen in dit onderzoek laten een veel lage degradatie snelheid zien vergeleken met aërobe condities; de gevonden specifieke *k*-waarden waren 0.10 en 0.006 l.g $TSS^{-1}.d^{-1}$ voor E1 en EE2 (Hoofdstuk 4 en 5).

Onder **anoxische** condities is de omzetting van E1 en E2 veel langzamer vergeleken met aërobe condities; de gevonden omzettingssnelheden voor EE2 lagen dicht bij de snelheden gevonden in de abiotische controles. Anoxische condities resulteerden in een lage afbraaksnelheid van E1 van ongeveer 0.004 l.g $TSS^{-1}.d^{-1}$ en afbraak van EE2 was afwezig, zelfs na de toevoeging van een substraat en her-enting. Fahrbach *et al.* (2006) isoleerde een gram negatieve denitrificerende bacterie uit actief slib die in staat was om E2 volledig te mineraliseren, maar informatie over de mogelijkheid tot EE2 degradatie is niet verkregen.

Resultaten van **anaërobe** korte termijn afbraakexperimenten uitgevoerd met actief slib, lieten geen afname in de som van de E1 en E2 concentraties zien; het dominante proces in deze experimenten was de reductie van E1 naar E2 (Hoofdstuk 4), hoewel er ook wat her-omzetting van E2 naar E1 waar te nemen was. Onder vergelijkbare omstandigheden werd er tevens geen afname van de EE2 concentratie gevonden, ook niet na toevoeging van substraat en her-enting (Hoofdstuk 5).

Resultaten van lange termijn (205-256 d) anaërobe afbraakexperimenten uitgevoerd met verschillende soorten slib, te weten vergiste varkensmest, granulair UASB slib, UASB septic tank slib en actief slib, lieten in overeenstemming met de korte termijn experimenten geen afname in de som van E1 en E2 zien. De snelheid en mate van de reductie van E1 naar E2 varieerden tussen de verschillende slibsoorten (Hoofdstuk 4). In vergelijkbare lange termijn experimenten werd ook geen afname in EE2 concentratie gevonden (Hoofdstuk 5), wat in overeenstemming is met resultaten van Czajka and Londry (2006) die ook geen EE2 afbraak vonden in geënte sedimenten en oppervlaktewater batch experimenten onder anaërobe condities over een periode van 3 jaar, zelfs niet in aanwezigheid van de oxidanten NO_3^-, SO_4^{2-} of Fe^{3+}. Dit is in tegenstelling tot de resultaten van Carballa *et al.* (2006) die een verwijdering van 85±5% en 75±15% rapporteerden voor EE2 in een continu slib vergister onder respectievelijk mesofiele en thermofiele condities en na een slib adaptatie periode (duur niet gerapporteerd). Het verschil met de observatie in dit onderzoek blijft onduidelijk en vraagt om opheldering tijdens toekomstig onderzoek. Een interessant punt is dat actief slib na een lange tijd van 200 dagen onder anaërobe condities te zijn geweest, nog steeds in staat is om EE2 af te breken zodra er zuurstof in de testfles komt (Hoofdstuk 5); dit zou kunnen duiden op een rol van facultatieve aërobe bacteriën bij de afbraak.

7'.4 Gedrag van oestrogenen in conventionele RWZI's

Biodegradatie snelheden en adsorptie constanten voor oestrogenen verkregen met behulp van batch experimenten kunnen worden gebruikt om effluentconcentraties in actief slib systemen voor het behandelen van huishoudelijk afvalwater te berekenen. Gemiddelde effluent oestrogeen concentraties uit de in de literatuur gerapporteerde waarden, zoals gemeten in praktijk RWZI's (Hoofdstuk 2) bedragen: 13.9±14.3 ng/l voor E1, 2.1±2.7 ng/l voor E2 en 1.7±4.3 ng/l voor EE2. De berekende gemiddelde verwijderingpercentages waren 62±27%, 88±9% en 56±24% voor respectievelijk E1, E2 en EE2. Systemen met nitrificatie en denitrificatie leiden tot de beste verwijderingen van E1, E2 en EE2.

Adsorptie en biodegradatie van oestrogenen in een actief slib systeem kunnen gemodelleerd worden met een methode zoals aangedragen door Temmink (2001), te weten Vergelijking 1. Het actief slib systeem is hierbij verondersteld zich als een continue, volledig gemengde reactor te gedragen. De gekozen proces parameters zijn gebaseerd op gemiddelde waarden voor conventionele actief slib systemen met nitrificatie/denitrificatie.

$$C_e = \frac{Q_i \cdot C_i}{\frac{1 + K_d \cdot X_a}{1 + K_d \cdot X_e} \cdot (Q_w + V_a \cdot k \cdot X_a) + Q_i - Q_w} \qquad \text{Vergelijking 1}$$

Waarin:

C_e: Concentratie van componenten in het effluent na de actief slib behandeling (ng/l)

C_i: Gemiddelde concentratie van de componenten in het influent van de actief slib behandeling (ng/l)

X_a: Biomassa concentratie in het actief slib systeem; waarde: 4 g TSS/l, meest toegepast in conventionele RWZI's

X_e: Biomassa concentratie in het effluent; geschat op 0.02 g/l (aanname)

Q_i: Debiet van de vloeibare fase; uitgaand van 10000 inwoner equivalenten (1 inwoner equivalent= 136 g CZV.person^{-1}.d^{-1}), droog weer afvoer: 1300 m^3/d, regen weer afvoer: 2000 m^3/d.

Q_w: Debiet van het spuislib; berekening gebaseerd op een slib retentietijd (SRT) van 12 d, welke een gemiddelde waarde is voor een actief slib systeem met nitrificatie/denitrificatie (Andersen *et al.*, 2003)

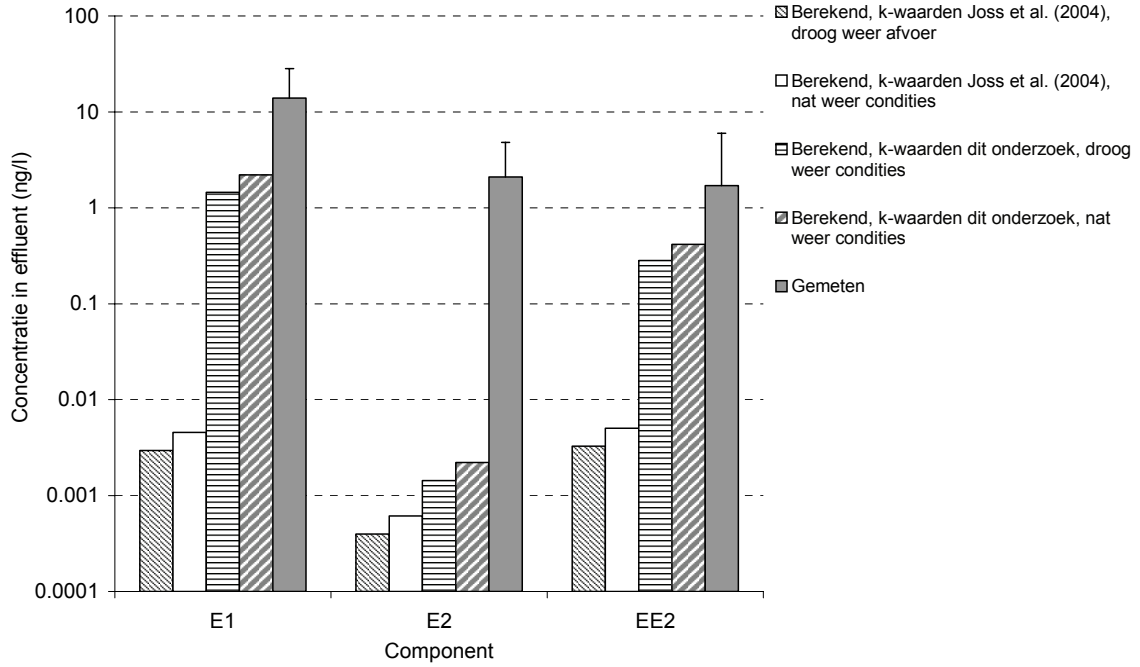
V_a: Volume aëratietank (m^3); berekend met gemiddelde slibbelasting van 0.25 kg CZV.kg TSS^{-1}.d^{-1} (Metcalf and Eddy, 2003); 1360 m^3

k: Specifieke eerste orde degradatie constante, Tabel 7'.2 (l.g TSS^{-1}.h^{-1})

K_d: Partitie coëfficiënt vast-water in gesuspendeerd materiaal; E1: 0.402 l/g TSS, E2: 0.476 l/g TSS, EE2: 0.584 l/g TSS (Andersen *et al.,* 2005)

Resultaten van de model berekeningen zijn weergegeven in Figuur 7'.1. Zowel de relatief lage specifieke eerste orde degradatie constante uit dit onderzoek, als de hoge waarden zoals gevonden door Joss *et al.* (2004) zijn gebruikt in de berekeningen. De gemiddelde gemeten effluent waarden voor praktijk behandelingssystemen voor huishoudelijk rioolwater (Hoofdstuk 2) zijn ook weergegeven in Figuur 7'.1. De gemiddelde gemeten

effluentconcentraties worden beter voorspeld bij gebruik van de lage specifieke eerste orde degradatie waarden (*k*-waarden) zoals gevonden dit onderzoek. De hogere gemeten oestrogeen concentraties in het effluent zouden veroorzaakt kunnen worden door het feit dat RWZI's in praktijk geen volledig gemengde systemen zijn. Hierdoor kunnen zones met een zuurstof deficiëntie en kortsluitstromingen/kanaalvorming voorkomen en deze laatste reduceren de werkelijke hydraulische retentietijd. Daarnaast kan de omzettingssnelheid gelimiteerd zijn door een verminderde biobeschikbaarheid als een resultaat van bijvoorbeeld snelheidsbeperkende desorptie. Verder kan de influentconcentratie van oestrogenen onderschat zijn doordat in veel gevallen de fractie geadsorbeerd aan deeltjes niet meegenomen is en door onvoldoende informatie over de fractie geconjugeerde oestrogenen in RWZI influenten. Hun rol zou nog wel eens groter kunnen zijn dan tot op heden gedacht.



Figuur 7'.1. Gemodelleerde en gemiddelde gemeten waarden voor concentraties van E1, E2 en EE2 in RWZI effluenten (logaritmische schaal), bepaald met gemiddelde gemeten influentconcentraties en eerste orde degradatie constanten (*k*-waarden) uit Joss *et al.* (2004) uitgevoerd met lage initiële concentratie oestrogene dosering en *k*-waarden uit dit onderzoek (Vergelijking 1). Gemeten waarden zijn gemiddelden van eind effluent waarden (Hoofdstuk 2).

7'.5 Gedrag van oestrogenen in innovatieve zwartwater behandelingssystemen

7'.5.1 Anaërobe voorbehandeling

Van een op semitechnische schaal bedreven UASB septictank systeem met een influent bestaande uit zwartwater verzameld door vacuüm toiletten, werd in het effluent substantiële concentraties van E1 en E2 gevonden, te weten respectievelijk 4.02±0.52 µg/l en 18.69±10.04 µg/l. Deze waarden bestaan uit de som van zowel de geconjugeerde als de ongeconjugeerde componenten. De hogere gemeten concentratie voor E2 is in overeenstemming met het feit

dat E1 gereduceerd wordt tot E2 onder anaërobe condities (Hoofdstuk 4). Een aanzienlijk percentage van de natuurlijke oestrogenen aanwezig in het UASB effluent, was in de geconjugeerde vorm, te weten >70% voor E1 en >80% voor E2. Van het ongeconjugeerde E1 was 53% en 25% van het ongeconjugeerde E2 geassocieerd met deeltjes >1.2 μm. De hogere adsorptie van E1 aan deeltjes vergeleken met E2 was conform de gevonden K_f adsorptie waarden (Hoofdstuk4). EE2 kon niet worden gedetecteerd door de GC-MS in het UASB-septic tank effluent.

Of de deconjugatie van de 20-30% natuurlijke oestrogenen plaatsvond gedurende de anaërobe behandeling of tijdens het transport/opslag is niet bekend. Volgens D'Ascenzo *et al.* (2003) werd een kleine hoeveelheid glucuronideconjugaten gedeconjugeerd gedurende opslag in een septic tank, terwijl sulfaatconjugaten onveranderd bleven.

Op basis van de uitgevoerde degradatie testen van de natuurlijke en synthetische oestrogenen onder anaërobe condities (Hoofdstuk 4 en 5), wordt er geen degradatie verwacht in de UASB-septic tank. Omdat aangetoond is dat de oestrogenen vooral aanwezig zijn in de geconjugeerde vorm, welke goed oplosbaar zijn in water, zou ook adsorptie maar in beperkte mate kunnen optreden, zodat er in het anaërobe proces slechts een zeer beperkte verwijdering plaatsvindt. Met een verwachte totale concentratie van 42 μg/l in het UASB septic tank influent (De Mes and Zeeman, 2003) bij een spoelvolume van 0.5 L, zou de maximale verwijdering voor zowel E1 en E2 slechts 46% bedragen.

7'.5.2 Nabehandeling

In het eind effluent van de nabehandelingsystemen, bestaande uit twee zogenaamde "down hanging sponge" (DHS) reactoren in serie gevolgd door een zandfilter, zijn E1 en E2 nog steeds in een substantiële hoeveelheid aanwezig met een totale concentratie van respectievelijk 1.37±1.45 μg/l en 0.65±0.78 μg/l. Percentages van 77% en 82% van de gemeten ongeconjugeerde E1 en E2 waren geassocieerd met deeltjes (>1.2 μm) in het eind effluent (Hoofdstuk 6).

Wanneer het influent van de nabehandeling additioneel gedoseerd wordt met E1, E2, EE2 en het sulfaatconjugaat van E2, was de verwijdering >99% voor zowel E2 en EE2 en 83% voor E1; 40% van de E1 en 99% van de E2 was aanwezig in geconjugeerde vorm in het eind effluent. Omdat er geen geconjugeerde E1 was toegevoegd, kan deze waarde volledig toegeschreven worden aan de hoeveelheid die natuurlijk aanwezig is in zwartwater. In het algemeen worden geconjugeerde oestrogenen niet meebepaald in praktijk RWZI's (Hoofdstuk 2). Een opmerkelijke hoeveelheid is nog steeds aanwezig in de geconjugeerde vorm na anaërobe behandeling (70 en 80% voor E1 en E2) en na microaërobe nabehandeling (40% van E1). Dit is in overeenstemming met waarnemingen door Adler (2001) en Gentili *et al.* (2002) die laten zien dat geconjugeerde oestrogenen nog steeds een significante bijdrage leveren (meer dan 40%) aan de totale oestrogeen concentraties in de effluenten van RWZI's. Zowel de resultaten van de metingen met en zonder additionele oestrogeen doseringen laten zien dat deconjugatie niet zo snel plaatsvindt als in het algemeen was aangenomen.

De aanwezigheid van substantiële hoeveelheden van E1 en E2 in het eind effluent van de nabehandeling, maakt de toepassing van een additionele zuiveringsstap, zoals bijvoorbeeld

ozonatie, noodzakelijk. Volgens Escher *et al.* (2006) resulteert ozonatie van urine in een hoge verwijdering van oestrogenen (>99%) en is daarnaast effectief voor de verwijdering van een scala aan farmaceutica (Snyder *et al.*, 2005). Als andere effectieve nabehandelingsystemen voor de verwijdering van E1, E2 en EE2 kunnen nano- en utlrafiltratie (Yoon *et al.*, 2006), omgekeerde osmose en electrodialyse (Maurer *et al.*, 2006) en UV-behandeling (Snyder *et al.*, 2005), genoemd worden. Ook een membraan bioreactor kan genoemd worden als tertiaire behandeling, welke ook kan dienen als alternatief voor conventionele actief slib systemen. MBR slib gaf hogere afbraaksnelheden voor oestrogenen, maar in de praktijk zijn de waarden in het eind effluent waarden niet noodzakelijkerwijs lager (Hu *et al.,* 2007; Clara *et al.*, 2005). Dit kan veroorzaakt worden door de lagere hydraulische retentie tijd in een MBR, terwijl hogere slib concentraties kunnen worden toegepast, blijft de slibbelasting ongeveer gelijk. Urmenyi *et al.* (2005) toonde een goede mogelijkheid om specifiek oestrogenen te verwijderen met een membraan waarin antigenen zijn ingebouwd. Bij de huidige stand der techniek is deze optie op het moment nog veel te duur.

Het duidelijke voordeel van een brongescheiden sanitatie concept ten opzichte van een conventioneel sanitatie concept is dat bijna alle oestrogenen aanwezig zijn in een klein volume, namelijk in het zwartewater, evenals verscheidene andere door mensen uitgescheiden microverontreinigingen zoals farmaceutica. Daarnaast kan door het extreem kleine volume van 7 $l.p^{-1}.d^{-1}$, in vergelijking tot het volume van conventioneel huishoudelijk afvalwater van ≥ 130 $l.p^{-1}.d^{-1}$, het volume van de benodigde aanvullende behandeling ook vrij compact blijven.

7'.6 Concluderende opmerkingen en toekomstige onderzoeksperspectieven

Zoals uit voorgaande onderzoeken blijkt, is het synthetische oestrogeen 17α-ethynyloestradiol het meest persistent van de drie onderzochte stoffen in dit onderzoek. Hoewel een toenemende slib retentietijd een duidelijk positief effect had op de verwijderingsnelheid in actief en membraan bioreactor slib, laten de verkregen resultaten uit dit onderzoek zien dat het vermogen tot nitrificatie niet een vereiste is.

Voor de afbraak van alle drie onderzochte oestrogenen zijn aërobe redox condities duidelijk het meest voordelig. De resultaten van batch experimenten, uitgevoerd onder gecontroleerde laboratorium omstandigheden, kunnen niet direct vertaald worden naar het gedrag in praktijk installaties. In de praktijk wordt de verwijdering mede bepaald door andere processen dan biodegradatie alleen. Daarnaast zijn in dit onderzoek vrij hoge initiële oestrogen concentraties toegepast (meer dan 100 keer voor E1 en E2 en meer dan 1000 keer voor EE2), welke nooit in huishoudelijk praktijk afvalwater zullen voorkomen. Toch bleken de uitgevoerde experimenten geschikt om de verschillende toegepste condities te vergelijken als ook sneheidsbepalende stappen te bepalen. Dit onderzoek laat duidelijk zien dat snelheidsbeperkende desorptie van geadsorbeerde oestrogenen kan resulteren in een lagere afbraaksnelheid, en tevens in een mindere verwijdering.

De verzamelde data in het huidge onderzoek zouden een input kunnen zijn voor modelering om verdere relaties te vinden tussen de verschillende parameters die een invloed hebben op de afbraak in praktijk installaites, eventueel ondersteund door aanvullende experimenten.

Ook kan onderschatting van de influent oestrogenen concentratie bijdragen aan hoger dan verwachte effluentconcentraties op basis van afbreekbaarheidexperimenten. Onderschatting kan worden veroorzaakt door het feit dat er geen rekening wordt gehouden met oestrogenen die gebonden zijn aan deeltjes en door het gebrek aan kennis omtrent de bijdrage van geconjugeerde oestrogenen. Daarnaast is er geen informatie beschikbaar over het gedrag van de conjugaten onder de verschillende redox condities. De resultaten zoals gepresenteerd in dit proefschrift impliceren dat er slechts een gedeeltelijke deconjugatie van de natuurlijk oestrogenen oestron en 17β-oestradiol optreedt tijdens anaërobe behandeling. Er is een duidelijke behoefte aan opheldering over de mate van deconjugatie als ook het adsorptie gedrag van deze.

Terwijl RWZI effluenten beschouwd worden als voornaamste bron in de bijdragen van hormoonverstoorders aan oppervlaktewateren, kan de rol van mest niet geheel over het hoofd worden gezien. Lokaal zijn er hoge concentraties waargenomen in drainagewateren nabij met mest behandelde akkers (Kjær *et al.*, 2007).

De nieuwste ontwikkelingen op het gebied van DeSaR in Nederland staat bekend onder de naam "nieuwe sanitatie", en omvat de brongescheiden inzameling van urine (www.stowa.nl). Dit biedt een zeer goede oplossing voor het voorkomen van vervuiling met betrekking tot nutriënten (Wilsenach *et al.*, 2005; Wilsenach, 2006). Voorzichtigheid moet in acht worden genomen wanneer dit tevens als oplossing voor het voorkomen van de lozing van oestrogenen en farmaceutica wordt aangedragen. Gemiddeld wordt namelijk nog 30% van de farmaceutica uitgescheiden in de feces (Alder *et al.*, 2006) en het risico voor het milieu wordt voor urine en feces gelijk geschat (Lienert *et al.*, 2006). Lienert *et al.* (2007) vermoeden dat farmaceutica uitgescheiden in de feces in het algemeen meer hydrofoob zijn waardoor ze de neiging hebben om aan het slib te sorberen waardoor ze ook beter te verwijderen zijn dan de meer hydrofiele substanties in urine. In het geval van oestrogenen leidt een gezamenlijke behandeling van urine en feces tot het toenemen van de hoeveelheid hydrofiele componenten door deconjugatie. Meer onderzoek is noodzakelijk om hierover duidelijke conclusies te kunnen trekken.

De volledige verwijdering van oestrogenen in biologische behandelingssystemen noodzaakt de toepassing van een aanvullende zuiveringsstap. Al is de verwijdering van oestrogenen in de biologische behandelingssystemen voor de behandeling van zwartwater minimaal, heeft brongescheiden sanitatie nog steeds de voorkeur boven conventionele concepten gezien het feit dat alle oestrogenen en een verzameling van farmaceutica aanwezig zijn in een zeer klein volume. Dit biedt goede mogelijkheden voor de toepassing van compacte tertiaire behandelingsystemen, na de anaërobe en (micro-)aërobe behandelingsstappen. Tevens voorkomt de toepassing van brongescheiden sanitatie systemen de risico's voor contaminatie door overstorten volledig.

Chapter 8

References

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Appendices

Appendix I Glassware conditioning

In an additional test described below, the amount of estrogens adsorbed to the glassware was investigated for two additional pre-treatment steps: left in a silane over night, or solely rinsed with Millipore water.

Set-up

Glass culture tubes (used-ones, as adsorption is most likely due to small cracks in the glass) were first rinsed with methanol, dried and then subjected to pre-treated with either (1) silanised with 5% trimethylchlorosilane (TMS, Sigma, the Netherlands) in toluene (Aros, the Netherlands, pro analysi quality) and left over night or (2) with rinsing three times with Millipore water. Five solutions of E2 were prepared in the range of 20-2000 nMol (5.45-544.78 μg/L) in dimethylsulfoxide (DMSO Sigma, the Netherlands). The solutions were left in the pre-treated culture tubes overnight, after which the E2 concentrations were determined with a YES bioassay (Murk et al. 2002). E2 was chosen for this experiment as it can be directly related to the concentration in the YES bioassay, in which E2 is used as a standard reference (Murk et al. 2002). Environmental samples or samples containing other compounds are expressed as E2-equivalents.

Results

Table 3.1 presents the recovery of E2 added in different concentrations to the glass culture tubes which received the three different additional pre-treatments. In all three cases the highest applied concentration felt outside the reliable range for quantification. The recovery was the highest for the lowest concentrations, and generally recoveries above 80% were obtained, except for the two highest concentrations with TMS pre-treatment, where recovery was <70%. Pre-treatment with TMS gives a significantly lower confidence interval (no overlap) compared to rinsing with Millipore water (Table 3.1).

Table 3.1. Recovery and 95% confidence interval of E2 added in different concentrations, after pre-treatment of glassware with Millipore water, NaOH (1%) and TMS (5%).

Applied pre-treatment	Added concentration E2 (nM)	Measured concentration E2 (nM)	Recovery (%)
Millipore water	58.00	55.23	95
	116.00	101.79	88
	174.00	141.07	81
	232.00	204.07	88
	655.00	2384.99[a]	364[a]
95% confidence interval			82-94
TMS (5%)	52.50	44.83	85
	105.00	88.39	84
	157.50	111.81	71
	201.00	128.36	64
	1115.00	1249.54[a]	112[a]
95% confidence interval			66-86

[a] Outside reliable range of the detection method.

Appendix II N-mass balance E1 degradation experiments

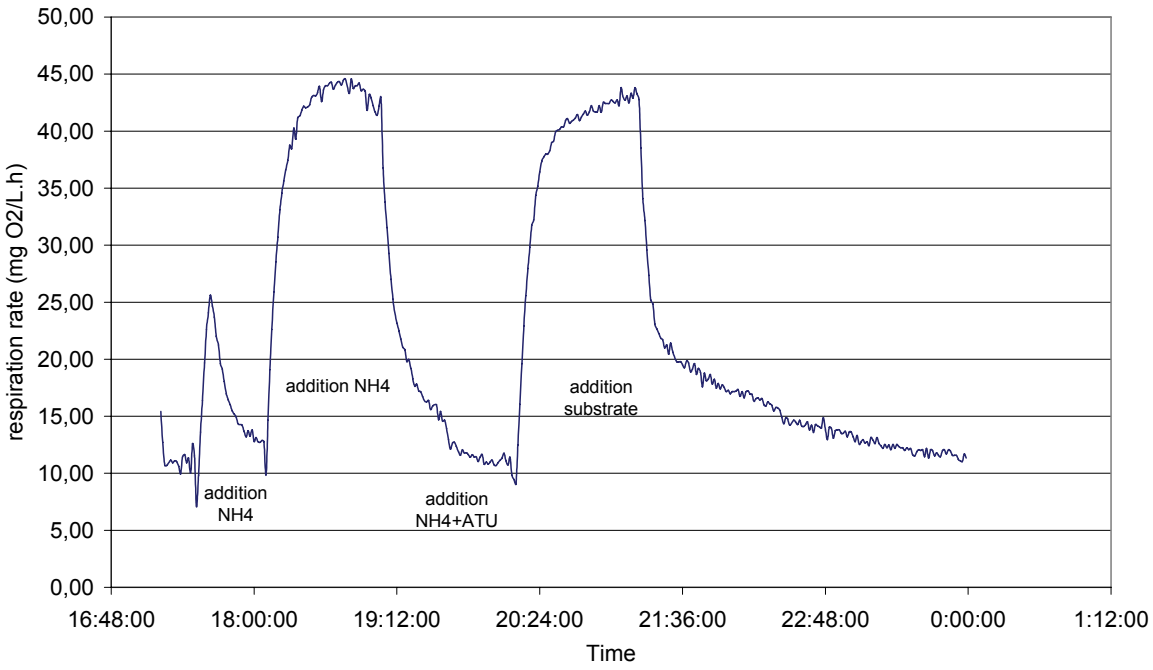
The assessed concentrations of N-compounds in paper filtered samples and the increase/decrease of their concentrations in relation to the initial concentration.

Experiment		NH_4^+ (mg N/l)	NO_3^- (mg N/l)	NO_2^- (mg N/l)	ΔNH_4^+ (mg N/l)	ΔNO_3^- (mg N/l)	ΔNO_2^- (mg N/l)	Δ N (mg/L)	NO_3^--N added to the anoxic batch (mg)	N_2-N formed (mg)
A	t_0[a]	4.53	6.97	0.37						
	Aerobic	12.62	63.3	0.39	8.1	56.3	0.02	64.4		
B	t_0[a]	4.53	6.97	0.37						
	Anaerobic	40.1	3.7	0.4	35.6	-3.3	0.03	32.33		
	Anoxic	153.0	35.0	5.0	148.5[b]	28.03	4.63	181.13	411.06	229.93
	Micro-aerobic	15.6	16.5	0.4	11.1	9.5	0.03	20.63		
	Aerobic	15.5	53.7	1.3	11.0	46.7	0.9	58.63		
	Aerobic ATU	48.4	10.8	0.6	43.9	3.8	0.2	47.93		
C	t_0	4.53	6.97	0.37						
	Aerobic	0.9	34.83	0.08	-3.6	27.9	-0.3	23.94		
	Aerobic+ ATU	17.58	2.99	0.05	13.1	-4.0	-0.3	8.75		
	Aerobic 2xdil.[c]	0.16	12.94	0.05	-4.4	6.0	-0.3	1.28		
	Aerobic 4xdil.[c]	0.22	2.99	0.04	-4.3	-4.0	-0.3	-8.62		

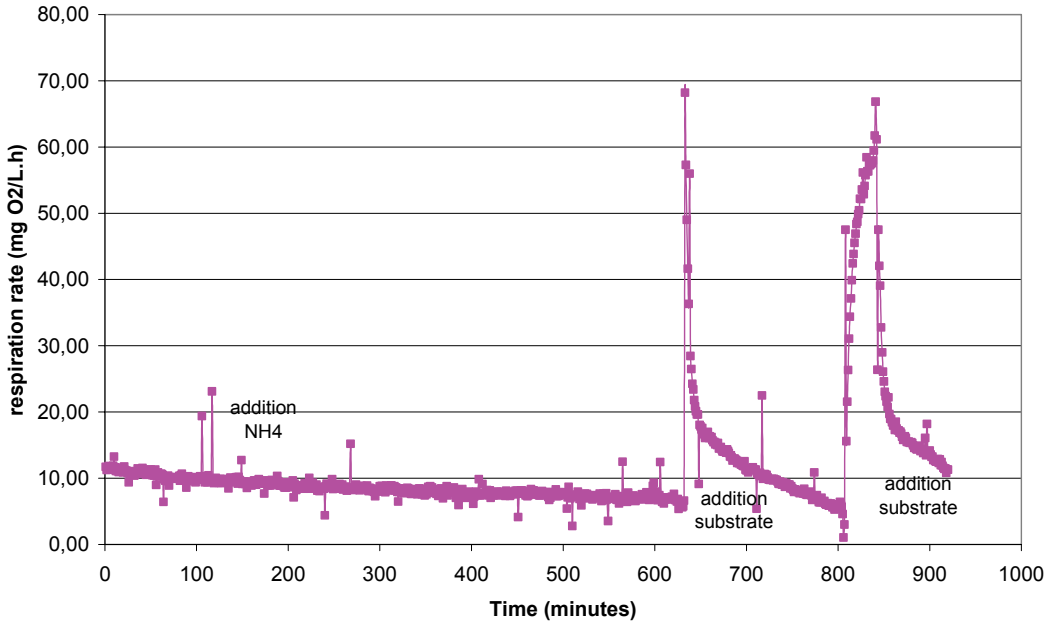
[a]t_0 measurements only done during experiment C, as the samples were always taken at the same location at the full scale plant, of which the operation was assumed to be stable. [c]dil.=diluted

Appendix III Respiration curves

Respiration curve of the activated sludge of the Bennekom STP at the start of the experiment.



Respiration curve of the Sequencing batch reactor (SBR)-sludge at the start of the experiment.



Appendix IV Environmental conditions

Environmental conditions in the batches spiked with EE2; average values determined on the sampling times and their standard deviations, and range for redox potential, VSS and TSS at t_0 of the experiment.

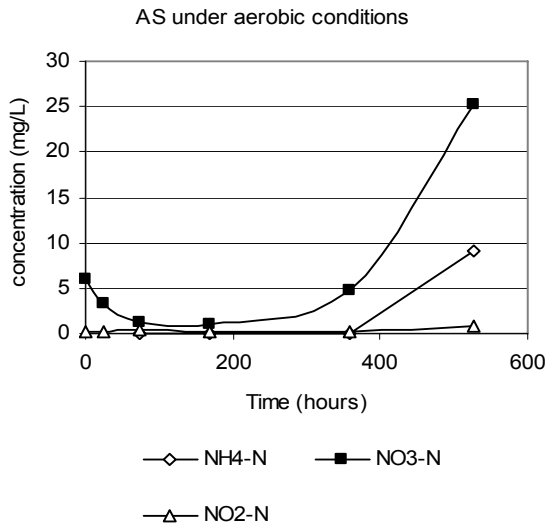
Experiment	Δtime (hrs)	DO (mg/l)	T (°C)	Redox potential (mV)	pH	Conduct-ivity ($\mu S.cm^{-1}$)	VSS_0 (g/l)	VSS_{end} (g/l)
A AS Aerobic	815	8.53±0.51	21.36±1.84	132 to 260	6.6±0.9	n.a.[a]	n.a.	1.26
AS Anoxic	435	n.a	24.82±1.73	46 to 202	7.5±0.7	n.a.	n.a.	1.50
AS Anaerobic	435	n.a.	24.83±1.85	-303 to -94	6.4±0.4	n.a.	n.a.	1.26
B AS Aerobic	476	8.56±1.11	23.77±3.13	77 to 224	6.5±0.5	n.a.	1.99	1.17
AS Anoxic	476	n.a.	25.63±2.89	-378 to -102	7.5±0.4	n.a.	1.99	1.47
AS Anaerobic	476	n.a.	26.14±3.44	77 to 224	6.1±0.4	n.a.	1.99	1.67
C AS Aerobic	834[b]/	8.81±0.87	19.02±0.90	40 to 292	6.3±1.6	598±366	1.86	1.35
AS Aerobic+ATU	744[c]	8.87±0.98	19.02±1.11	18 to 246	7.2±1.3	674±346	1.86	1.37
AS Micro-aerobic	834[b]/	0.73±1.21	21.76±0.91	-214 to 173	7.0±1.0	739±597	1.86	2.15
AS Anaerobic	744[c]	0.32±0.18	21.34±0.69	-428 to -273	6.4±0.4	972±827	1.86	1.40
D AS Aerobic [fraction 1]	331	7.47±0.24	29.10±0.41	59 to 152	8.3±0.1	600±74	0.03	0.006
AS Aerobic [fraction 2]	331	7.25±0.13	30.00±0.36	55 to 138	8.1±0.2	436±63	0.13	0.052
AS Aerobic [fraction 3]	331	6.65±0.93	30.59±0.02	71 to 176	7.2±0.5	534±106	1.58	0.67
AS Aerobic [fraction 4]	331	6.97±0.37	30.48±0.53	93 to 176	6.6±0.8	658±143	2.29	0.66
AS Aerobic [fraction 5]	331	6.55±1.04	30.14±0.67	106 to 192	6.2±0.9	770±223	3.69	1.39
E AS Anoxic	620	0.10±0.13	19.57±0.65	-394 to -87	7.7±0.7	2.15±1.21[d]	2.42	1.88
AS Anoxic + substrate	620	0.18±0.28	19.10±0.64	-359 to -126	8.3±0.8	4.68±2.72[d]	2.42	1.83
AS Anaerobic	620	0.16±0.24	19.64±0.59	-319 to -52	6.8±0.2	1.12±0.33[d]	2.42	2.06
AS Anaerobic + substrate	620	0.20±0.27	19.20±0.52	-350 to -64	6.9±0.1	3.66±1.69[d]	2.42	2.06
F AS Aerobic	670	7.46±2.77	18.76±0.37	48 to 235	6.8±0.6	397±90	1.61	0.96
AS Aerobic+ATU	670	8.51±0.95	18.71±0.45	51 to 145	7.5±0.3	398±114	1.61	1.26
SBR-S Aerobic	670	7.42±3.08	18.70±0.35	-8 to 125	7.7±0.6	712±90	0.56	0.14
G MBR-S Aerobic	498	9.07±0.23	18.73±0.38	57 to 174	8.4±0.2	845±25	1.36	1.22
H MBR-S Aerobic	772	9.14±0.41	17.04±2.6	133 to 194	8.3±0.1	978±9	1.74	1.46
I MBR-S Aerobic	506	6.88±1.03	24.81±2.17	203 to 273	8.4±0.3	38.86±0.94	2.11	1.53

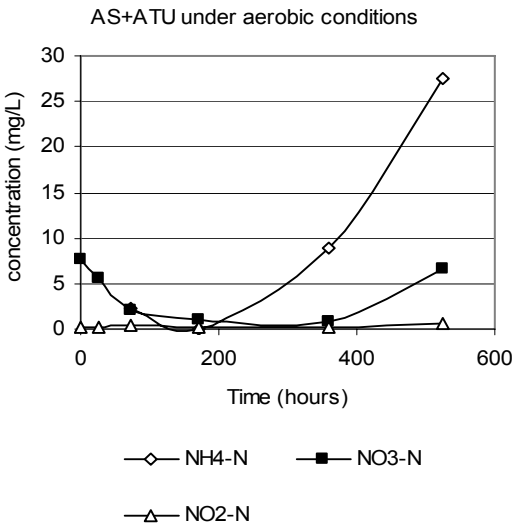
[a] not applicable [b]period before substrate addition [c]period when substrate was provided [d]in $mS.cm^{-1}$

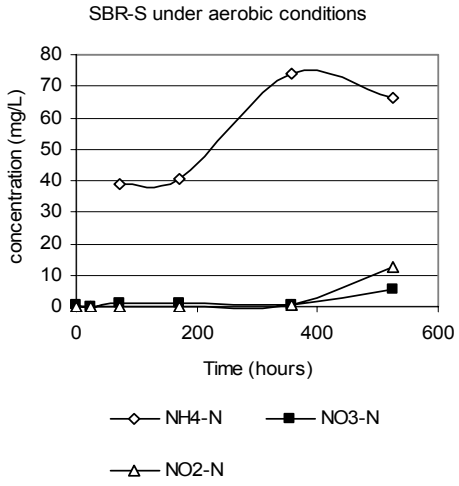
Appendix V N-mass balance EE2 degradation experiments

Nitrogen concentrations and mass balance for experiment F and experiment E. for ammonium, nitrate and nitrite in paper filtered samples, including the amount of nitrate added in the anoxic batch over the course of the experiment and actual measured concentrations in the batches of experiment F.

Experiment		NH_4^+ (mg N/l)	NO_3^- (mg N/l)	NO_2^- (mg N/l)	ΔNH_4^+ (mg N/l)	ΔNO_3^- (mg N/l)	ΔNO_2^- (mg N/l)	Δ N (mg/l)	NO_3^--N added (mg)	N_2-N formed (mg)
E	Anoxic unfed t_0	7.73	25.4	2.48						
	Anoxic unfed t_{end}	17.82	0.27	0.23	10.09	-25.13	-2.25	-17.29	884.33	901.62
	Anoxic fed t_0	7.93	32.78	2.9						
	Anoxic fed t_{end}	29.9	1.43	0.32	21.97	-31.35	-2.58	-11.96	884.33	896.29
F	Aerobic AS t_0	0	1.18	0.4						
	Aerobic AS t_{end}	9.04	25.3	0.76	9.04	24.12	0.36	33.52		
	Aerobic+ATU AS t_0	2.32	2.14	0.5	2.32					
	Aerobic+ATU AS t_0	27.6	6.72	0.7	25.28	4.58	0.2	30.06		
	Aerobic SBR t_0	38.98	1.12	0.26	38.98					
	Aerobic SBR t_{end}	66.06	5.48	12.56	27.08	4.36	12.3	43.74		







Appendix VI: First order degradation constants for EE2 degradation

Calculated *k*-values (with standard error) for the degradation of EE2 with the 95% confidence interval, calculated specific *k*-values on basis of TSS at the start of the experiment.

Exp.[a]	Sludge	Conditions	*k*-value (d^{-1})	95% confidence interval		TSS_0 (g/l)	Specific *k*-value (l.g TSS^{-1}. d^{-1})	Half-life (d/ gTSS)
				Lower value (d^{-1})	Upper value (d^{-1})			
A, B, C1 and F	AS	Aerobic	0.077±0.005	0.067	0.088	2.66	0.029	23.9
C2	AS	Aerobic 2nd spike+ substrate	0.034±0.012	0.007	0.058	1.79	0.019	36.5
C1 and F	AS	Aerobic +ATU	0.065±0.009	0.048	0.084	2.32	0.028	24.8
G	MBR-S	Aerobic	0.24±0.02	0.21	0.29	2.52	0.095	7.3
H	MBR-S	Aerobic	0.18±0.02	0.15	0.22	3.33	0.054	12.8
M	MBR-S	Aerobic	0.81±0.1	0.60	1.03	3.52	0.23	3
D	$AS^{fraction1}$	Aerobic	0.050±0.007	0.034	0.070	0.0049	1.02	0.7
D	$AS^{fraction1}$	Aerobic (corrected ambient temperature)	0.033±0.007	0.022	0.046	0.033	0.67	1.0
D	$AS^{fraction2}$	Aerobic	0.058±0.007	0.038	0.077	0.21	0.27	2.6
D	$AS^{fraction2}$	Aerobic (corrected ambient temperature)	0.038±0.007	0.025	0.050	0.21	0.18	3.8
D	$AS^{fraction3}$	Aerobic	0.096±0.007	0.079	0.120	2.18	0.044	15.8
D	$AS^{fraction3}$	Aerobic (corrected ambient temperature)	0.062±0.007	0.052	0.078	2.18	0.029	23.9
D	$AS^{fraction4}$	Aerobic	0.125±0.010	0.103	0.144	3.13	0.040	17.3
D	$AS^{fraction4}$	Aerobic (corrected ambient temperature)	0.082±0.010	0.067	0.094	3.13	0.026	25.7
D	$AS^{fraction5}$	Aerobic	0.144±0012	0.110	0.175	4.80	0.030	23.1
D	$AS^{fraction5}$	Aerobic (corrected ambient temperature)	0.094±0.012	0.072	0.114	4.80	0.020	34.7
C	AS	Micro-aerobic	0.014±0.003	0.008	0.020	2.55	0.0055	126.0
B and E[b]	AS	Anoxic	0[c]	0	0	1.73	0	∝
A, B, C and E[d]	AS	Anaerobic	0[c]	0	0	1.77	0	∝
F	SBR-S	Aerobic	0.192±0.046	0.094	0.29	0.69	0.28	2.5

[a]exp.=experiment, [b]including data for substrate addition and second spike [e]approaching zero

Appendix VII: Parameters two compartmental approach degradation EE2

Calculated degradation parameters of EE2 with the two compartmental approach; for the fraction that is degraded rapidly (F_{rapid}) and slowly (F_{slow}) with their resembling first order degradation constants (k_{rapid} and k_{slow}).

Experiment	Sludge	Conditions	F_{rapid}	F_{slow}	k_{rapid} (d^{-1})	k_{slow} (d^{-1})
A, B, C1 and F	AS	Aerobic	0.51	0.49	0.19	0.041
C2	AS	Aerobic 2nd spike+substrate	0.04	0.96	15.88	0.032
C1 and F	AS	Aerobic +ATU	0.30	0.70	1.58	0.050
G	MBR-S	Aerobic	0.80	0.20	0.34	0.066
H	MBR-S	Aerobic	0.17	0.83	5.80	0.15
I	MBR-S	Aerobic	0.13	0.87	76.15	0.19
D	AS fraction1	Aerobic	n.a.[a]	n.a.	n.a.	n.a.
D	AS fraction1	Aerobic (corrected ambient temperature)	n.a.	n.a.	n.a.	n.a.
D	AS fraction2	Aerobic	n.a.	n.a.	n.a.	n.a.
D	AS fraction2	Aerobic (corrected ambient temperature)	n.a.	n.a.	n.a.	n.a.
D	AS fraction3	Aerobic	0.14	0.86	0.04	0.004
D	AS fraction3	Aerobic (corrected ambient temperature)	0.14	0.86	0.026	0.003
D	AS fraction4	Aerobic	0.05	0.95	1.32	0.005
D	AS fraction4	Aerobic (corrected ambient temperature)	0.05	0.95	0.86	0.003
D	AS fraction5	Aerobic	0.16	0.84	3.98	0.005
D	AS fraction5	Aerobic (corrected ambient temperature)	0.16	0.84	2.60	0.003
C	AS	Micro-aerobic	0.08	0.92	10.18	0.013
B and E[b]	AS	Anoxic	n.a.	n.a.	n.a.	n.a.
B, C and E[b]	AS	Anaerobic	n.a	n.a.	n.a.	n.a.
F	SBR-S	Aerobic	0.58	0.42	0.85	0.055

[a]not applicable [b]including data for substrate addition and second spike

Curriculum Vitae

Titia Zita Dolly de Mes, born on 22nd of February 1974 in Haarlem, the Netherlands, obtained in 1992 the "Atheneum" diploma at the Albanianea in Alphen a/d Rijn. This diploma gave her access to the Wageningen University and Research (WUR), where she studied environmental sciences with specialisation environmental technologies for developing countries. After a graduation research in the field of wastewater treatment, her skills were put in practice during a six-month internship in the Philippines at the Water laboratory and Chemical engineering department of the University of San-Carlos in Cebu-city, which was in cooperation with the Centre for International Cooperation of Delft University of Technology. After a second graduation research on soil remediation, she successfully finished the MSc in 1997.

After graduation she was still involved at WUR, organising lectures and supervising groups of students, before she started working in 1998 as environmental consultant on soil remedation at the Blgg, laboratory for agriculture, in Oosterbeek the Netherlands. At the end of 1999, she started to work for the Lettinga Associates Foundation (LeAF) as site engineer based in Accra, Ghana working together with Taysec Construction Ltd. (daughter company of the British Taylor Woodrow) where she was responsible for the start- up of the newly-built central sewage treatment works. In a later stage she assisted in the commissioning trials. In between stays in Ghana, she was working at LeAFs' headquarters in Wageningen, as consultant performing numerous projects. In order to satisfy her hunger for in depth knowledge on wastewater treatment, she started her PhD-research at the sub-department of Environmental Technology at WUR for four days a week, while still committed to LeAF for one day a week. Towards the end 2002, Titia took a four month leave to take the role of site-chemist to the treatment plant related laboratory in Accra. After this she was fully committed to her Ph.D. research of which the results are presented in this thesis.

At present she is working on the Dutch, but internationally oriented consultancy firm, DHV as (waste)water specialist.

Publications and Supervised Researches

Full papers

De Mes T., Hyde R. and Hyde K. (2003a). Anaerobic first for Ghana. *Water21*: 30-31.

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Acknowledgements

Past six years a lot has happened, though the time seems to have shot by. Even though a Ph.D. research is most of all a bit of a lonely occupation, many people crossed my path who I like to acknowledge.

First of all I would like to address my co-promotor Grietje Zeeman, who was closely involved at all stages of the research and gave me the opportunity to work on this matter as part of the multi-disciplinary Decentralised Sanitation and Reuse (DeSaR) project financed by the Dutch government. I believe that multi-disciplinary, or even better interdisciplinary research, has many advantages and besides the research field, originally "Fate of pharmaceuticals their metabolites and pathogens in wastewater treatment and their consequences for DeSaR" definitely draw my attention. The subject was in 2001 new, challenging and I was sure I would be able to dedicate myself to it for a five-year period. Even with narrowing the subject down to the fate of estrogens, and even only three of them, five-years seemed a bit too short for this complicated matter. My other motivation to commit myself to this research was that I am fully supporting the DeSaR concept. Why diluting wastewater, when we could even gain energy and regain nutrients if treat it undiluted in an anaerobic digester? With the role Gatze Lettinga has played in making anaerobic digestion popular for treating concentrated wastewater, I am very honored that he is my promotor, even though he retired just before I started. After a long list of promovendi, I am the last one and it is a bit unfortunate that the UASB-septic tank used during this research did not prove itself to be able to substantially remove the estrogens. My other co-promotor, Katarzyna Kujawa-Roeleveld, was involved in setting up the whole DeSaR project and is now extensively working on the fate of pharmaceuticals. I would like to thank the three of you for all your support, guidance, flexibility, but also for the pleasant informal gatherings.

My gratitude goes out to Katja Grolle who was involved at the very start of the research helping me to overcome my first thresholds towards the analytical determination and again at the final stage, assisting me with my final experiments and checking the whole dissertation. Due to the complexity of the subject not a lot of students dared to do a research on this matter. However, I would like to thank Elena Cordoba, Darja Kragić, Weijun Chen and Xia Xia Zhang for being my heroes! I hope you learned as much from me as I did from you. Darja, thank you very much for also working with me on this matter after your graduation on a fellowship grant and for being my personal assistant in the last difficult days and above all being a great friend! I would like to thank my partners in crime Hellen Elissen and Bas Buys, unfortunately after the closing down of our beloved experimental hall Bennekom things got a bit unbalanced. Hellen, we need to get back into our weird-dancing routine soon, hopefully we will pick it up when we both finished with the, how it seemed, never ending commitment to our thesis. I have got a lot of nice memory's to the Bennekom time, with all the Bennekom

people! People of the department with a clear practical or scientific input/discussions like Martijn, Tim, Jasperien, Vinnie, Hilion, Ilse, Bert W., Bert H., Harry, Hardy, Liesbeth, Gusta as well as people outside the department Tinka (Toxicology, WUR), Herlinde (Ghent University), Bert (Rikilt), all my officemates and people from COST action 636 (Xenobiotics in the urban environment), your input was very much appreciated.

To my colleagues at DHV, who witnessed the bags under my eyes grew into scary sizes last few weeks before the submission of my thesis: thank you for the warm welcome, the pleasant working atmosphere and the support. I really felt at home straight from the start.

And for the last, but not the least I would like to thank all my family and friends, for being there for me! Special thanks to my dear neighbors, for all the good care! And Simon, thank you for your love and putting my feet back on the ground whenever this was required. It reaches too far to mention everybody by name, but I did organize a party for all of you where I will express my gratitude in person!

With this research I hope I have contributed to increased knowledge on the fate of estrogens in biological treatment systems. As often happens in research, many new questions arose. Therefore I would like to encourage future researches to fill up these gaps and to continue where I left.

This fish is heading for open water now, hopefully it is without any estrogenic pollution!

Titia de Mes
22nd September 2007

Picture taken at the M.Sc. graduation of Weijun, from left to right: Darja, Weijun, me, Xia Xia.

This thesis describes research that was supported by the Dutch government in the Economy, Ecology and Technology (EET) programme, being part of the multidisciplinary "Decentralised Sanitation Reuse" project with the aim of investigating and facilitating the applicability of the concept in practice.