Fate, pathways and methods for the determination of selected antibiotics and steroid hormones in the environment

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Summary

Pharmaceuticals are mainly introduced into the environment via two pathways. The first path is the application of veterinary drugs to animals, as they are excreted, high concentration are found in manure. As this contaminated manure is utilised for fertilising the fields a soil issue arises. The second path is the treatment of infections in the human medicine. When these residues are excreted they are transported through the sewers to sewage treatment plants and are then discharged with the treated wastewater into the aquatic environment. In this thesis the enviromental fate of the antibiotics erythromycin, roxithromycin, clarithromycin, tylosin, oleandomycin, tiamulin, salinomycin, the steroid hormones 17 β-estradiol, estrone, estriol, 16 α-hydroxyestrone and β-estradiol 17-acetate, the hormone-conjugates β-estradiol 3-sulfate and estrone 3-sulfate, the oral contraceptives 17 α-ethinylestradiol and mestranol were studied.

To assess the fate of the macrolide antibiotics, ionophores, pleuromutilins, steroid hormones, oral contraceptives and hormone-conjugates, three new analytical methods were developed in respect of the different matrices manure, soil and wastewater. These analytical methods are based on the extraction methods liquid liquid extraction (LLE), solid phase extraction (SPE) and accelerated solvent extraction (ASE) in combination with the clean-up steps SPE, size exclusion chromatography (SEC) and the detection in different ionisation modes of high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Isotopic labelled internal standards were used to account for matrix-effects in the HPLC-MS/MS analysis. As the availability of deuterated macrolide antibiotics standards is poor, a new macrolide internal standard was synthesised. For the determination of veterinary pharmaceuticals the limit of quantifications (LOQ) were determined. They were between 1.4 and 11 ng/g for manure and 0.6 to 30 ng/g in soil, whereas the LOQ in wastewater ranged from 0.6 to 35 ng/L . For the analysis of the steroid hormones and macrolide antibiotics in wastewater compliance within EU decision 657/2002/EC was achieved.

The fate of the veterinary used antibiotics was studied with degradation experiments under anaerobic and aerobic conditions in manure and soil. Half-lives for antibiotics in manure ranged from 5 days up to >200 days and half-lives in soil ranged from 5 days up to >120 days. Additionally new metabolites of the antibiotic salinomycin in manure were identified by means of high performance liquid chromatography with electrospray ionisation coupled with high resolution time of flight mass spectrometry (HPLC-ESI-HR-TOF-MS) and different tandem mass spectrometric techniques.

The fate of steroid hormones and macrolide antibiotics during wastewater treatment was researched by testing the elimination efficiencies of three different concepts of STPs over four weeks at different weather conditions. While larger STPs eliminated hormones more constantly than smaller STPs, heavy rainfall events led to a collapse of the biological treatment step. By using trickling filter techniques for the treatment of wastewater an elimination of the steroid hormones could not be observed. Also no significant elimination of macrolide antibiotics during wastewater treatment could be detected in all three STPs.

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Abbreviations and acronyms

-	value < Limit of detection
*	value < Limit of quantification
amu	atomic mass unit
APCI	atmospheric pressure chemical ionisation
ASE	accelerated solvent extraction
$b_{antibiotic}$	concentration of antibiotics in manure
BLAC	Bund/Länderausschluss für Chemikaliensicherheit
c	concentration
c_0	start concentration
C	addition of dissolved organic carbon
CAD	collision gas
CAS	chemical abstract registry number
CE	collision energy
CLA	clarithromycin
C_{org}	organic carbon
cps	counts per second
CUR	curtain gas
d_n	n-fold deuterated
DP	declustering potential
E1	estrone
E1S3	estrone 3-sulfate
E2	17 β-estradiol
E2Ac	β -estradiol 17-acetate
E2S3	β -estradiol 3-sulfate
E3	estriol
EDTA	ethylenediaminetetraacetic acid, disodium salt
EE2	17 α-ethinylestradiol
EP	entrance potential
ERY	erythromycin
ESI	electrospray ionisation
EU	European Union
FP	focusing potential
GC	gas chromatography
GS1	ion source gas 1
GS2	ion source gas 2
h	horizontal arrangement
H_{depth}	penetration depth of the antibiotics in soil

HE1	16 α-Hydroxyestrone
HPLC	high performance liquid chromatography
HR-MS	high resolution mass spectrometry
hum	human medicine
IC	ion spray voltage
IEV	inhabitant equivalent value
ihe	interface heater
IS	internal standard
IS-A	internal standard for the analysis of macrolide antibiotics
IS-H	internal standard for the analysis of the steroid hormones
k	kinetic factor
LOD	limit of detection
LOQ	limit of quantification
ME	mestranol
min	minute
MRM	multi reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	monoisotopic molecular mass
m/z	mass to charge ratio
N	neutralisation line
n.v.	not validated
na	not available
NC	nebuliser current
N_{org}	organic nitrogene
NRW	North-Rhine-Westphalia
P	phosphate precipitation
PEC	predicted environmental concentration
PTFE	polytetrafluoroethylene
q	MRM transition used for quantification
q_0	pre-quadrupole
Q_N	area based load of manure
R^2	regression coefficient
ROX	roxithromycin
rpm	rounds per minute
RSD	relative standard deviation
RT	retention time
s	second
S/N	signal to noise ratio
SD	standard deviation

SEC	size exclusion chromatography
SPE	solid phase extraction
SRM	selected reaction monitoring
STP	sewage treatment plant
t	time
$t_{1/2}$	half-life
TEM	temperature
THF	tetrahydrofurane
TOF-MS	time of flight mass spectrometer
va	vertical arrangement
v	MRM transition used for verification
v/v	volume weighted
vet	veterinary medicine
w/w	mass weighted
δ	chemical shift
ρ_{manure}	density manure
ρ_{soil}	density soil

List of publications

Publications in peer reviewed journals

Schlüsener M.P., Bester K.; Persistence of antibiotics such as macrolides, tiamulin and salinomycin in soil Environ. Poll. 143 (2006) P. 565-571

Schlüsener M.P., von Arb M., Bester K.; Degradation of antibiotics such as macrolides, tiamulin and salinomycin in liquid manure. Arch. Environ. Contam. Toxicol. 51 (2006) P.21–28

Schlüsener M.P., Bester K.; Determination of steroid hormones, hormone conjugates and macrolide antibiotics in influents and effluents of sewage treatment plants utilizing HPLC-MS/MS with ESI and APCI. Rapid Comm. Mass Spec. 19 (2005) P.3269-3278

Schlüsener M.P., Spiteller M., Bester K.; Determination of antibiotics from soil by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1003 (2003) P.21-28

Schlüsener M.P., Bester K., Spiteller M.; Determination of antibiotics such as macrolides, ionophores and tiamulin in liquid manure by HPLC-MS/MS. Anal. Bioanal. Chem. 375 (2003) P.942-947

Poster presentations

Schlüsener M.P., Bester K.; Elimination of hormones and antibiotics during wastewater treatment. Comparison of three different STPs, SETAC-Europe, 7.5.-11.5.2006, The Hague, Netherlands

Schlüsener M.P., Bester K.; Analysis of steroid hormones, steroid hormone conjugates, and pharmaceuticals from wastewater, SETAC-Europe, 22.5.-26.5.2005, Lille, France

Schlüsener M.P., Bester K.; Abbau von Veterinärpharmaka (Makrozyklen, Polyether, und Pleuromutiline) in landwirtschaftlichen Böden, UBA-Meeting, 29.9.-30.9.2004, Berlin, Germany

Schlüsener M.P., Bester K.; Degradation of macrolides, salinomycin and tiamulin in soil, SETAC-Europe, 18.4.-22.4.2004, Prague, Czech Republic

Schlüsener M.P., Bester K., Spiteller M.; Determination of macrolides and tiamulin in complex matrices, Euroanalysis 12, 8.9.-13. 9.2002, Dortmund, Germany

Schlüsener M.P., Bester K., Pfeifer T., Spiteller M.; Determination of polyether antibiotics and tiamulin from manure and soil samples, SETAC-Europe, 12.5.-16.5.2002, Vienna, Austria

Platform presentations

Bester K., Schlüsener M.P., Pfeifer T., Spiteller M.; Methods to determine macrocyclic antibiotics in manure and soil, ACS, 8.4.-13.4. 2002, Orlando, United States

Schlüsener M.P., Bester K., Spiteller, M.; Accelerated solvent extraction (ASE) and clean-ups for HPLC-MS/MS analysis; PreConference Course, Euroanalysis 12, 8.9.-13.9.2002, Dortmund, Germany

Schlüsener M.P., Bester K., Spiteller, M.; Degradation of macrolides, salinomycin and tiamulin in liquid manure; SETAC-Europe Conference, 27.4.-1.5. 2003, Hamburg, Germany

Bester K., Schlüsener M.P.; Antibiotika in Abwasser, Gülle und Klärschlamm, 3rd Colloquium „Anwendung der LC-MS in der Wasseranalytik“; 3.6.-4.6.2003, Berlin, Germany

Spiteller M., Schlüsener M.P. Bester K.; Determination of Antibiotics in Manure, Soil and Sewage Sludge; 3rd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals in Water; 17.5.-18.5.2003 Minneapolis, United States

Schlüsener M.P.; Steroid-Hormone und Makrozyklische Antibiotika - Gründe für die Verweiblichung von Fischen; Fachseminar, Bestimmung von Abbaubilanzen von Substanzen aus Personal Care Produkten und Hormonen in Kläranlagen; 18.10.2005, Universität Duisburg-Essen, Germany

Schlüsener M.P.; Antibiotika in Gülle und Böden; Vorkommen, Eliminierung und Transformationsprodukte; GDCh Neujahrskolloquium, 11.01.2006, Universität Duisburg-Essen, Germany

Schlüsener M.P., Bester K.; Elimination and transformation of selected antibiotics in soil, manure, and waste water; Pittcon 2006, 12.3.-17.3.2006 Orlando, Florida, USA

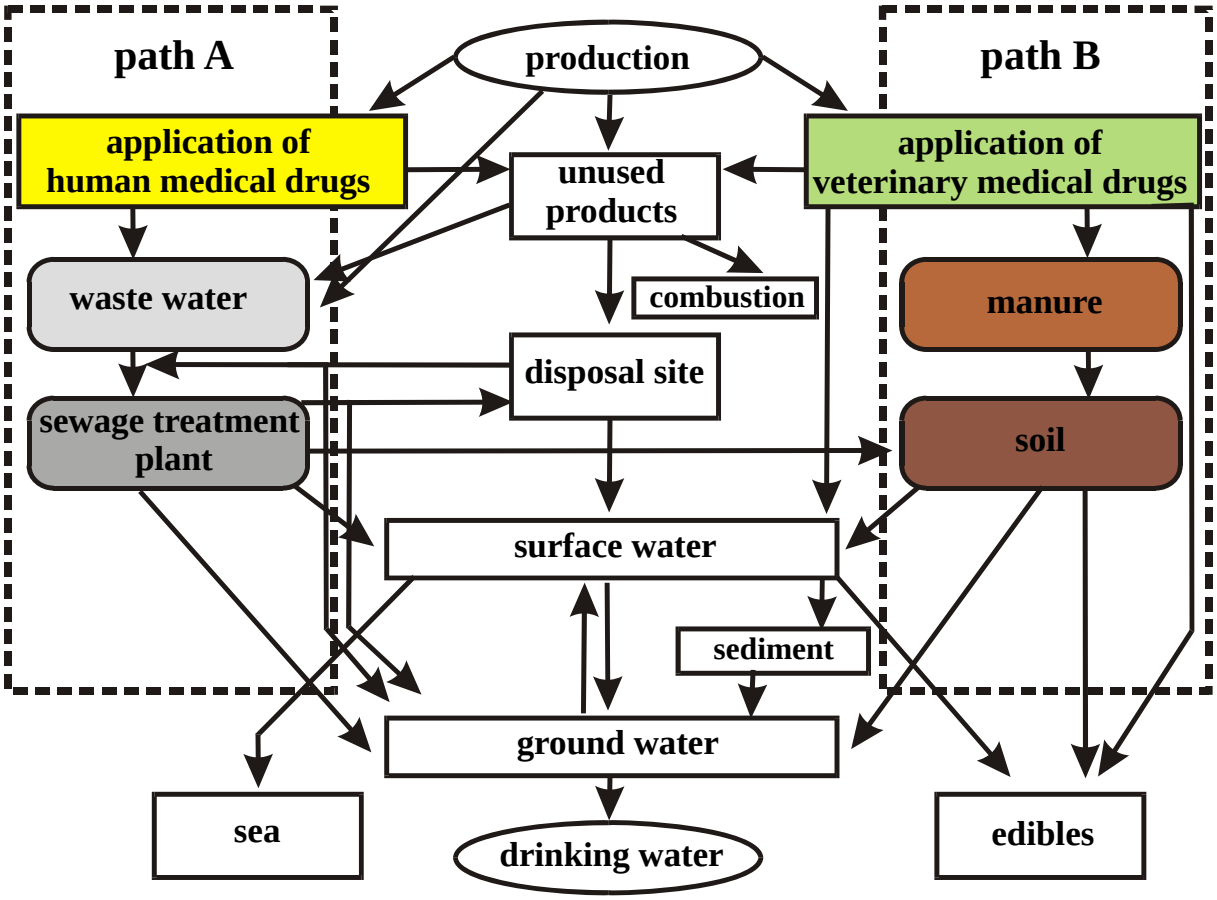
1 Introduction

Over 2,900 different medical substances are registered in Germany [1]. These pharmaceuticals are administered orally, intravenously or applied onto the skin depending on the compound itself and the medical circumstances. The fate of these drugs following application were long time neglected. Only when different drugs were detected in the environment [2, 3, 4], toxic effects on fauna, flora and environmentally relevant bacteria has been observed [5, 6, 7, 8, 9] and a resistance of bacteria to the majority of existing antibiotics were detected [10, 11] it came to a rethinking in policy and society.

1.1 Pathways of pharmaceuticals into the environment

Pharmaceuticals enter the environment by different routes (Figure 1). Two paths are prevalent. Pharmaceuticals used in human medical medicine (path A) are excreted metabolised or unmetabolised via the urine and faeces shortly after administration [12].

Figure 1: Sources, distribution and sinks of pharmaceuticals in the environment according to Kümmerer [13]



These substances enter the sewage treatment plants via municipal sewage systems. If these drugs and their metabolites are not eliminated during the wastewater treatment processes, they enter the aquatic environment. Pharmaceuticals have been detected in several German rivers [14, 15, 16]. Also Weigel *et al.* and Buser *et al*. found clofibric acid, a metabolite of clofribrate, in the German Bight of the North Sea [17, 18, 19]. Pharmaceuticals could be even detected in German drinking water, as well as in groundwater [20, 21, 22].

A similar issue has risen in industrial agriculture. Veterinary drugs used in animal husbandry for the treatment and prevention of infections or to promote the weight of the animals [23]. They are excreted unchanged or metabolised with the manure (path B). After storing the manure several months in manure tanks, farmers use this stored manure to fertilise their fields. Also digested sewage sludge is used to fertilise fields. Thus the drug residues which are contained in the manure and sludge are introduced to the soil. Heavy rainfalls may wash these drug residues from the soil and they may thus enter the aquatic environment. Also antibiotics can inhibit the biogas production during the anaerobic digestion of liquid manure [24]. Additionally veterinary drug residues can enter edibles e.g. residues in eggs and meat. The food monitoring is part of the national food and drug administration and sometimes international affairs are taking place. In 2002 the European Union stopped the import of Chinese shrimps because of high levels of chloramphenicol, an antibiotic, in these shrimps [25, 26].

This work was performed to understand the environmental occurrence and fate of drugs. The first part focuses on the exposition route of veterinary drugs (path B), the occurrence and fate of veterinary used antibiotics in manure and soil. The second part of the work describes the exposure and fate of human medical drugs like steroid hormones and antibiotics in different sewage treatment plants (path A). As no methods for the determination of these drugs in these three matrices existed, new analytical methods had to be developed for the determination of veterinary and human drugs as well as steroid hormones in manure, soil and wastewater.

1.2 Choice of the analytes

As representative analytes for the veterinary path (B), the veterinary used antibiotics erythromycin, tylosin, oleandomycin, salinomycin and tiamulin were chosen. Tiamulin and salinomycin were used by the cooperating farmers. Additionally roxithromycin, at present exclusively used in human medicine, was chosen because roxithromycin may be approved for veterinary medicine applications in future. As sulfonamides and tetracyclines were actually studied by other groups these antibiotics were excluded in this work.

The behaviour of pharmaceuticals at the human path (A) is described by the steroid hormones 17 β-estradiol, estrone, estriol, 16 α-hydroxyestrone and 17 β-estradiol 17-acetate, the hormone-conjugates β-estradiol 3-sulfate and estrone 3-sulfate, the oral contraceptives 17α-ethinylestradiol and mestranol and the common used macrolide antibiotics erythromycin, roxithromycin and clarithromycin.

The subsequent tables show the structural and empiric formula, the monoisotopic mass (MW), the chemical abstract registry number (CAS) as well as the consumption of these compounds. The consumption of the veterinary used (vet) drugs tiamulin, erythromycin and tylosin, is based on the data of Broll *et al.* for Schleswig-Holstein [27]. This dataset was extrapolated to the whole of Germany by the assumption that Schleswig-Holstein's pig fattening is only 5 % of those of Germany. These data stems from the Federal Statistical Office of Germany [28].

Data on the consumption of the human used (hum) antibiotics and hormones in Germany is derived from the report of "Bund/Länderausschuss für Chemikaliensicherheit (BLAC), Arzneimittel in der Umwelt" as far as available [29]. The usage and sources into the environment of the analytes were compiled by using books about pharmacology [30], the drug catalog 'Rote Liste' [1] and a food chemistry encyclopaedia [31].

Table 1: Erythomycin

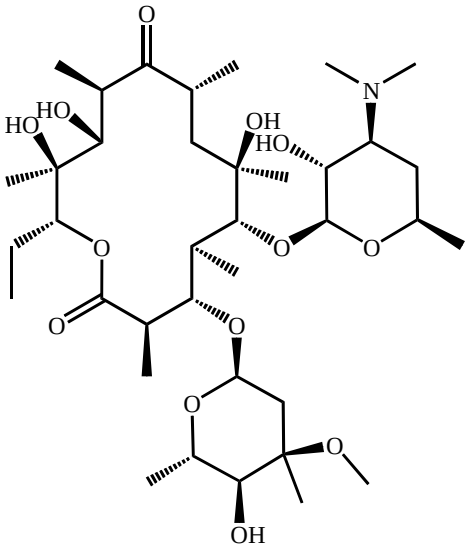
Macrolide antibiotic	
	Empirical formula: $C_{37}H_{67}NO_{13}$ MW: 733.46 CAS [111-07-8] Consumption: hum: 19 tons vet: 20 kg
Usage: In human medicine for the treatment of infections like scarlatina, tonsillitis, erysipelas, pneumonia, diphtheria, pertussis, acne vulgaris and for the prevention of the rheumatic fever. In veterinary medicine for the treatment of intestinal infection, mastitis and pneumonia.	

Table 2: Oleandomycin

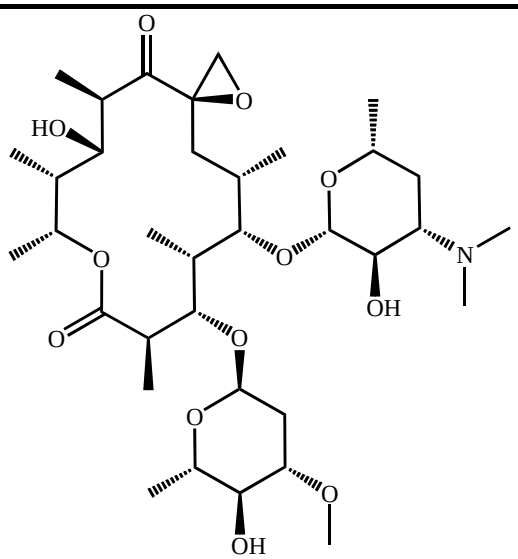
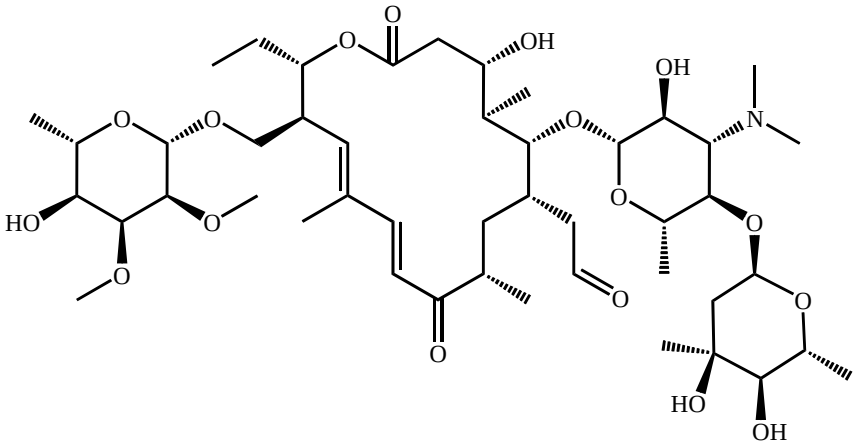
Macrolide antibiotic	
	Empirical formula: $C_{35}H_{61}NO_{12}$ MW: 687.42 CAS [7060-74-4] Consumption: hum: na vet: na
Usage: Oleandomycin is used in human and veterinary medicine. It has a similar application range as erythromycin, but with smaller effects. Primarily used for the treatment of mastitis and skin diseases in veterinay medicine. Mostly used as oleandomycinphosphate.	

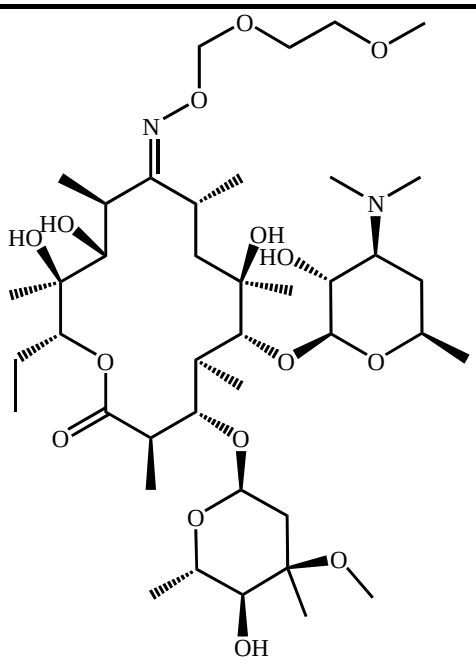
Table 3: Tylosin

Macrolide antibiotic	
	Empirical formula: $C_{46}H_{77}NO_{17}$ MW: 915.52 CAS [1401-69-0] Consumption: hum: - vet: 6.4 tons

Usage:

Treatment of dysentery at pigs as well as feed additive to promote the growth of pigs until 1998. Mostly used as tylosintartrate.

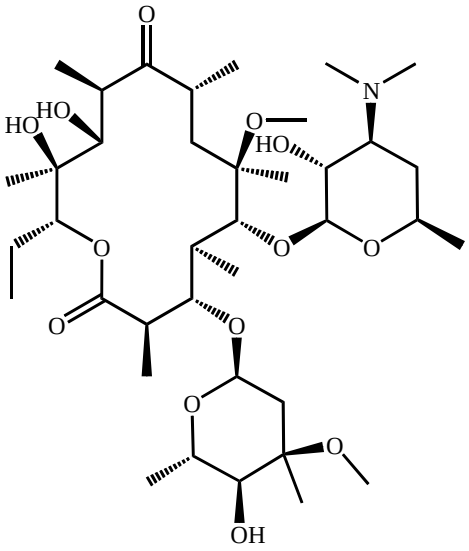
Table 4: Roxithromycin

Macrolide antibiotic	
	Empirical formula: $C_{41}H_{76}NO_{15}$ MW: 836.52 CAS [80241-83-1] Consumption: hum: 9.5 tons vet: -

Usage:

This oxime derivative of erythromycin has a similar application range as erythromycin, but it is only used in human medicine.

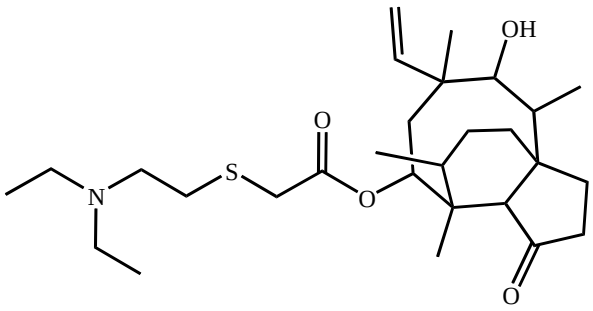
Table 5: Clarithromycin

Macrolide antibiotic	
	Empirical formula: $C_{38}H_{69}NO_{13}$ MW: 747.48 CAS [81103-11-9] Consumption: hum: 7.2 tons vet: -

Usage:

This methylether derivative of erythromycin has a similar application range as erythromycin but it is only used in human medicine.

Table 6: Tiamulin

Pleuromutilin derivative	
	Empirical formula: $C_{28}H_{47}NO_4S$ MW: 493.32 CAS [55297-96-6] Consumption: hum: - vet: 9.2 tons

Usage: This pleuromutilin derivative is only used in veterinay medicine for the prevention and treatment of the enzootic pneumonia, dysentery and sinusitis. Mostly used as the water soluble tiamulin hydrogen fumarate, tiamutin®.

Table 7: Salinomycin

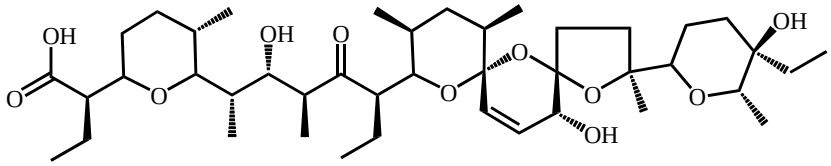
Ionophore	
	Empirical formula: $C_{42}H_{70}O_{11}$ MW: 750.49 CAS [53003-10-4] Consumption: hum: - vet: na
Usage: Salinomycin is used to promote the growth of pigs and as feed additive for the prevention and treatment of the coccidiosis. Mostly used as the water soluble sodium salinomycin.	

Table 8: 17 β-Estradiol

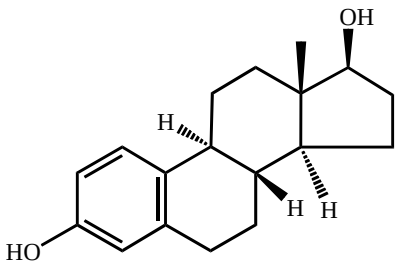
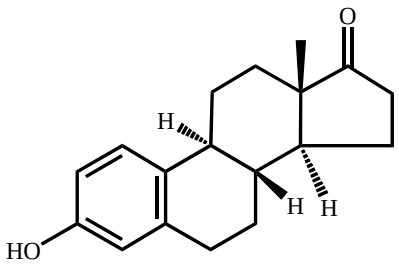
Steroid hormone	
	Empirical formula: $C_{18}H_{24}O_2$ MW: 272.18 CAS [50-28-2] Consumption: hum: 1.1 tons vet: -
Usage: Hormonal treatment for ailments in the climacteric period of women and prevention of the osteoporosis. 17 β-Estradiol is a natural steroid hormone and mainly excreted in the urine of mammals.	

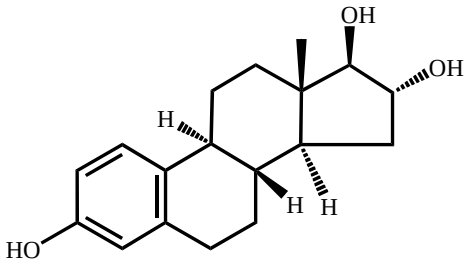
Table 9: Estrone

Steroid hormone	
	Empirical formula: $C_{18}H_{22}O_2$ MW: 270.16 CAS [53-16-7] Consumption: hum: 0 kg vet: -

Sources:

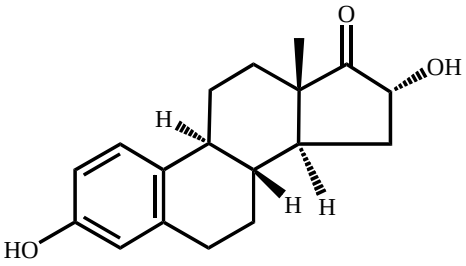
Estrone is a metabolite of estradiol and mainly excreted with the urine of mammals.

Table 10: Estriol

Steroid hormone	
	Empirical formula: $C_{18}H_{24}O_3$ MW: 288.17 CAS [50-27-1] Consumption: hum: 160 kg vet: -

Usage: Hormonal treatment for ailments during the female menopause and prevention of the osteoporosis. Estriol is a metabolite of estradiol and mainly excreted with the urine of mammals.

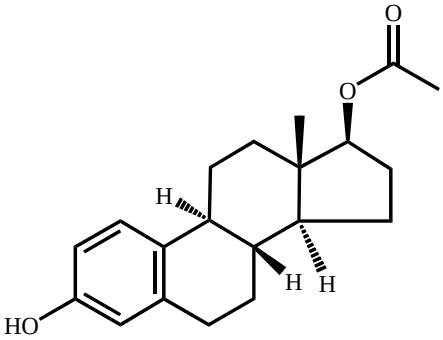
Table 11: 16 α-Hydroxyestrone

Steroid hormone	
	Empirical formula: $C_{18}H_{22}O_3$ MW: 286.16 CAS [566-76-7] Consumption: hum: - vet: -

Sources:

16 α-Hydroxyestrone is a metabolite of estradiol and mainly excreted with the urine of mammals.

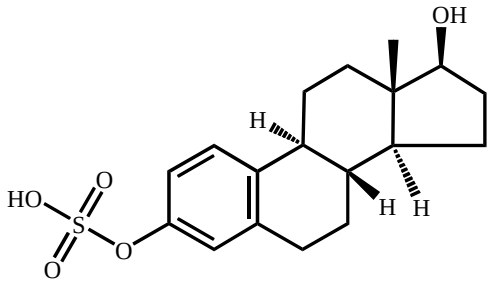
Table 12: β-Estradiol 17-acetate

Steroid hormone	
	Empirical formula: $C_{20}H_{26}O_3$ MW: 314.19 CAS [1743-60-8] Consumption: hum: - vet: na

Usage:

Hormonal treatment for ailments during the female menopause and prevention of the osteoporosis.

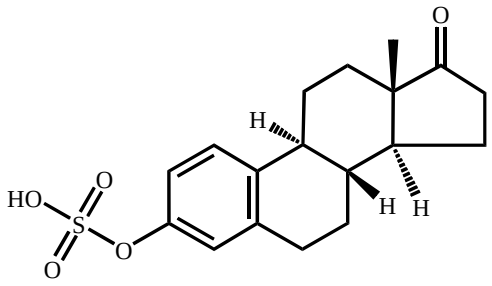
Table 13: β-Estradiol 3-sulfate

Hormone-conjugate	
	Empirical formula: $C_{18}H_{24}O_5S$ MW: 352.13 CAS [481-96-9] Consumption: hum: - vet: -

Sources:

β-Estradiol 3-sulfate is a conjugate of estradiol, formed in the bile and mainly excreted with the urine of mammals.

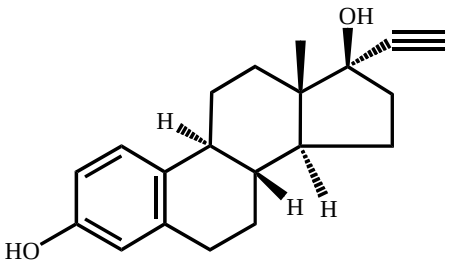
Table 14: Estrone 3-sulfate

Hormone-conjugate	
	Empirical formula: $C_{18}H_{22}O_5S$ MW: 350.12 CAS [481-97-0] Consumption: hum: - vet: -

Sources:

Estrone 3-sulfate is a metabolite of estradiol and a conjugate of estrone, formed in the bile and mainly excreted with the urine of mammals.

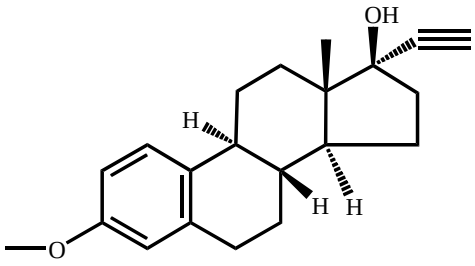
Table 15: 17 α-Ethinylestradiol

Oral contraceptive	
	Empirical formula: $C_{20}H_{24}O_2$ MW: 296.18 CAS [57-63-6] Consumption: hum: 50 kg vet: na

Usage:

Primary used for the oral contraception in human medicine also used for the contraception in veterinary medicine (pet care). Not used in the European industrial animal husbandry.

Table 16: Mestranol

Oral contraceptive	
	Empirical formula: $C_{21}H_{26}O_2$ MW: 310.19 CAS [72-33-3] Consumption: hum: 0.8 kg vet: -

Usage:

Primary used for the oral contraception in human medicine also used for the contraception in veterinary medicine (pet care). Not used in the European industrial animal husbandry.

2 Determination and elimination of selected antibiotics in liquid manure

2.1 Introduction to antibiotics in manure

Most of the 2,900 pharmaceuticals registered in Germany were used in animal husbandry as well as in human medical applications [1]. In the recent years the occurrence of these pharmaceuticals in the agricultural environment has been reported [15, 32, 33]. Additionally, the occurrence of antibiotics in influents, effluents and sludge of sewage treatment plants [34, 35] and surface waters [14, 36] has been discussed. Toxic effects on fauna have been observed as well [5]. Furthermore, bacterial resistance to the majority of existing antibiotics were first reported by Neu [10].

Over 10,000 tons of antibiotics were applied in Europe in 1997 as antibacterial agents. About 50 % is used in human medicine while the other half is applied in large-scale animal husbandry [37]. The veterinary antibiotic classes such as tetracyclines, sulfonamides, macrolides, ionophores and pleuromutilins are commonly used to treat infections in livestock. Three different uses of antibiotics are currently considered significant in, e.g., pig farming.

1.) The treatment of infections; e.g. with pleuromutilins and macrolides,
2.) Disease prevention; especially if pigs from different breeders are brought together for fattening, e.g. pleuromutilins and macrolides
3.) Growth promotion; which is the continuous dosing of an antibiotic compound, such as salinomycin, to pigs in order to promote growth during the fattening phase. Sodium-monensin, sodium-salinomycin, flavophospholipol and avilamycin are currently used for growth promotion in agriculture, but these antibiotics will probably be phased out in the EU on January 1st, 2006 [38]. Sodium-salinomycin is still allowed as feed additive for pigs until October 2009 [39] and for the prevention of coccidiosis until August 2014 [40].

A typical pig farm in Germany holds an average of 800 pigs [28]. Depending on the actual infections in the livestock, about 1-5 kg of each of the antibiotics are used during the fattening phase on such a farm. The pigs quickly absorb most of the compounds and excrete 50-90% (ß-lactam-antibiotics, tetracyclines, sulfonamides) of the initial amounts after several days [12]. The respective parent compound as well as their primary metabolites are prevalent in

excretions. Thus, large quantities of the pharmaceuticals are transferred to manure tanks, along with the liquid manure. After the storage, the manure is dispersed on the fields and the unmetabolised antibiotics contained in this manure may contaminate the soil and eventually the ground water. The occurrence and fate of veterinary drugs such as sulfonamides and tetracyclines in soil or manure has been reported [41, 42, 43]. Basic considerations about the fate of veterinary drugs have also been published by Tolls [44].

However, little is known about the fate of antibiotics in manure. In this medium they may undergo diverse reactions resulting in complete or partial elimination of the parent compound [45]. Three different degradation behaviours are possible:

1) Complete elimination by mineralisation of the antibiotics.

2) Partial transformation.

3) Persistence of the compounds in the manure.

These processes can be performed by biotic or abiotic means.

While the published literature focuses on sulfonamides and tetracyclines, the aim of this study was the development of a reproducible and sensitive multiresidue method to investigate the commonly used macrolides erythromycin, roxithromycin, and oleandomycin, the ionophore salinomycin as well as the pleuromutilin derivative tiamulin in liquid swine manure. Furthermore this study focuses also on the fate of these antibiotics during storage of liquid manure until the manure is spread on agricultural fields.

Manure may be stored about 180 days before being dispersed on fields [46] and is most often stored in anaerobic lagoons or storage tanks. Manure storage in ‘anaerobic lagoons’, typically used in the United States and Canada, are large outdoor basins of liquid manure, with air and sunlight on the surface but anaerobic in depth. Therefore, some oxygen may diffuse into the lagoon and sunlight may cause photo-transformations of antibiotics at the surface.

Manure storage tanks are more commonly used in Europe. In a manure tank, manure is stored in the dark and has reduced air admittance. In these tanks there are more anaerobic conditions than in lagoons.

2.2 Method development and validation

2.2.1 Experimental to antibiotics in manure

2.2.1.1 Chemicals to antibiotics in manure

Acetonitrile (HPLC-S gradient grade) was purchased from Biosolv (Valkensward, Netherlands). Water (HPLC grade) was obtained from Mallinckrodt Baker (Griesheim, Germany). Isooctane, methanol (Suprasolv grade), acetone, ethyl acetate, methylenchloride, chloroform, triethylamine, ammonium acetate, ammonium hydroxide, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, urea, disodium ethylenediaminetetraacetate, sodium sulfate, and calcium carbonate (analytical grade) were obtained from Merck (Darmstadt, Germany). Erythromycin, roxithromycin and O-methylhydroxylamine hydrochloride were provided by Sigma-Aldrich (Seelze, Germany). Oleandomycin phosphate dihydrate, sodium salt, salinomycin SV sodium salt 2.5-hydrate, tiamulin fumarate (Vetranal^{TM}) and sodium chloride (analytical grade) were obtained from Riedel-de Haën (Seelze, Germany).

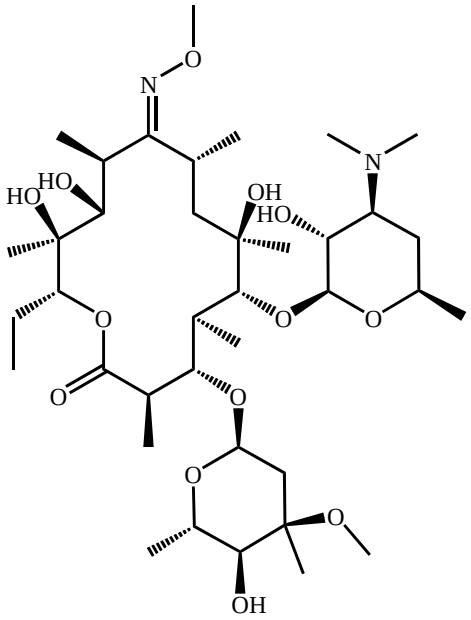
2.2.1.2 Internal standard

The synthesis of (*E*)-9-[O-(2-methyloxime)]-erythromycin was similar to the procedure described by Gasc *et al.* [47].

1,052 mg of calcium carbonate and 415 mg of O-methylhydroxylamine hydrochloride were added to a solution of 707 mg erythromycin in dry methanol and the mixture was stirred at room temperature for 96 hours. This solution was poured into 50 mL of an 5 % ammonium hydroxide solution and the resulting mixture was cooled in an ice-water bath. The mixture was extracted thrice with 30 mL methylene chloride.

The crude product was purified by chromatography on silica gel 60 with a chloroform : triethylamine mixture (9:1, v/v). The HPLC-MS separation of the derivative revealed a purity of 92% at mass 763.5. MS conditions were full scan from 150 to 1000 amu, device parameters are described in 2.2.1.7. No erythromycin was detected. The ^{1}H-NMR signals of the modification (300 MHz, $CDCl_3$) reveal δ 3.83 ppm (s, N-OCH_3) and δ 3.33 ppm (s, 4''-OCH_3). This is in agreement with the data from the literature [47]. This new macrolide was used as internal standard (IS-A). Figure 2 shows the structural formula of the new internal standard.

Figure 2: Structural formula of the new internal standard, (*E*)-9-[O-(2-methyloxime)]-erythromycin



2.2.1.3 Sample pre-treatment

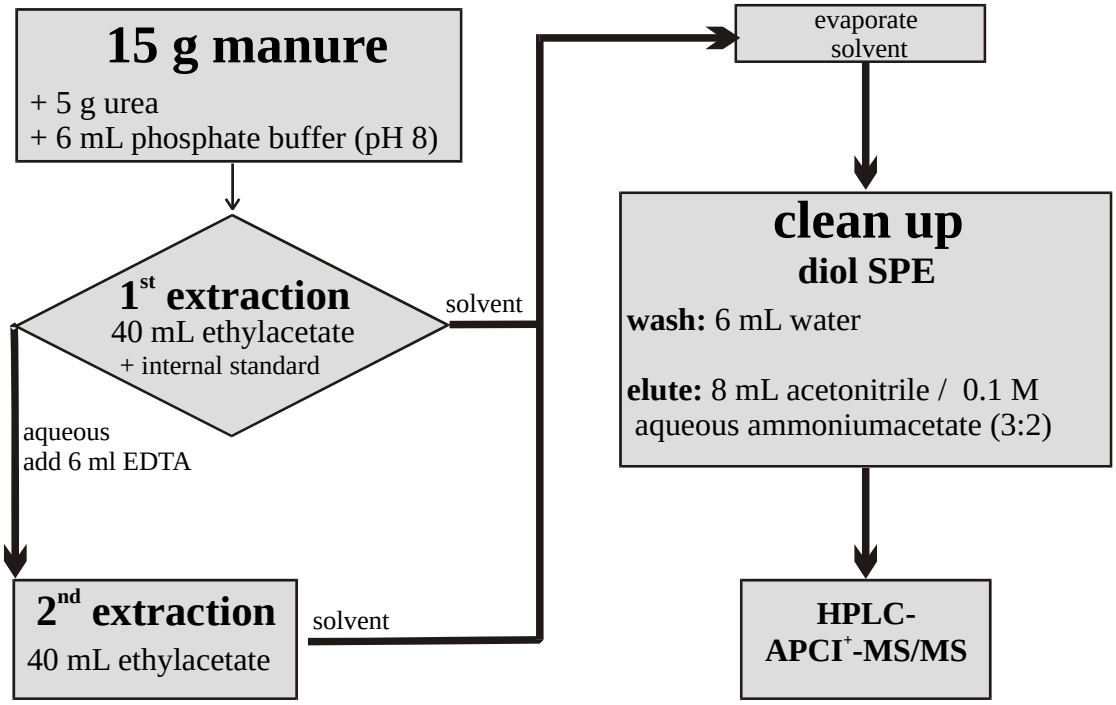
The manure was homogenised for 5 min at 25,000 rpm using an ultra turrax homogeniser (VF2 / IKA, Staufen, Germany). 15 g of homogenised manure were transferred into 75 mL centrifuge glass tubes with a screw cap (Schott, Mainz, Germany) and 5 g urea was added. The samples were buffered to pH 8 by the addition of 6 mL phosphate buffer (3.4 g K_2HPO_4, 0.1 g KH_2PO_4 dissolved in 100 mL HPLC-grade water).

2.2.1.4 Liquid-liquid extraction

The buffered manure was extracted with 40 mL ethyl acetate by shaking for 20 min on a horizontal shaker (Kottermann, type 4020, Haenigsen, Germany) at 150 min^{-1}. After shaking, before phase separation, 25 µL of internal standard solution (10 mg (*E*)-9-[-O-(2-methyloxime)]- erythromycin dissolved in 100 mL acetonitrile) was added to the mixture and the centrifuge glass was shaken by hand for 1 min. The phases were separated by centrifugation at 800 g (1,350 rpm) for 20 min (BeckmannCoulter, Avanti J25, Unterschleissheim, Germany). The organic phase was removed and stored. The aqueous phase was mixed with 6 mL EDTA solution (3.7 mg disodium ethylenediaminetetraacetic acid dissolved in 100 mL HPLC-grade water) and the mixture was extracted again with 40 mL ethyl acetate, with shaking (20 min) and centrifugation (800 g for 20 min). The organic

phases from both extractions were combined and the sample volume was reduced to 5 mL by means of a rotary evaporator at 60 °C and 320 mbar. The residue was dissolved in 20 mL isooctane and the volume was reduced again to 10 mL at 60 °C and 170 mbar. Figure 3 shows the procedure for analysis of antibiotics in manure.

Figure 3: Sample preparation manure



2.2.1.5 SPE clean-up

Samples were cleaned up by a modification of the method developed by Delépine *et al.* [48]. Diol solid-phase extraction cartridges from UCT (2,000 mg, Bristol/PA, USA) were conditioned once with 10 mL isooctane at a flow rate of 5 mL/min. A solid-phase extraction manifold (IST, Grenzach-Wyhlen, Germany) with PTFE stopcock and outlet, was used. The manure extract (10 mL) was passed through the cartridge at a speed of 5 mL/min (vacuum). The cartridge was washed once with 10 mL isooctane to remove lipids and dried for 20 min by sucking air through the column followed by a wash step with 10 mL water to remove salt. The analytes were eluted twice from the cartridge with 4 mL (3/2, v/v) mixture of acetonitrile:0.1 M aqueous ammonium acetate solution at a flow rate of 5 mL/min. An aliquot of 0.8 mL of the eluate was transferred to a 1.5-mL autosampler vial for HPLC-MS/MS analysis.

2.2.1.6 HPLC

The HPLC separations were performed using a Phenosphere-Next RP18 column (2 mm i.d., length 150 mm, particle size 3 µm) and a SecurityGuard (Phenomenex, Torrance, CA, USA) at 25 ± 1 °C. The flow rate was 0.2 mL/min. The HPLC gradient was established by mixing two mobile phases: phase A, 10 mM aqueous ammonium acetate solution and phase B, pure acetonitrile. Chromatographic separation was achieved with the following gradient: 0-1 min 10% B, 1 min to 14 min 10% to 100% B, 14-29 min 100% B, 29 min to 30 min 100% to 10% B, 30-35 min 10% B. Ten µL of each sample were injected.

The HPLC system consisted of a GINA 50 autosampler, a P 580A HPG HPLC pump, a degasser unit DEGASYS DG-1210 and a column oven STG 585 (all from Dionex, Idstein, Germany). The dead time of the HPLC system was 1.8 min.

2.2.1.7 Mass spectrometry

The triple quadrupole mass spectrometer (TSQ 7000, Finnigan-MAT, Bremen, Germany) was equipped with an APCI 2 source and operated under the following conditions: capillary temperature, 180 °C; sheath gas, 40 psi; corona current, 5 µA; vaporizer temperature, 450 °C; auxiliary gas, off; q_0 offset, -4.4 V; collision cell pressure, 2.0 mTorr; collision gas, argon; multiplier, 1900 V. The potential difference between the capillary and the tube lens was held at 70 V. The cycle time was 1.0 s during the chromatographic determination of antibiotics. The data obtained were processed using Xcalibur™ 1.2 software. The silica capillary of the APCI 2 source was replaced by a steel capillary in order to reduce tailing of antibiotics adsorbing on the silica surface [49]. While the electrospray ionisation is vulnerable to matrix effects, APCI was preferred [50, 51].

A post-column Valco divert valve was used to direct most of the non-significant HPLC flow of a sample to waste. Diverting the flow minimised contamination of the MS source: 0-8 min divert to waste, 8-28 min flow to mass spectrometer, 28-35 min divert to waste. An additional flow of 50 µL/min water acetonitrile (3:7, v/v) pumped by a LC-10 AT HPLC (Shimadzu, Duisburg, Germany) compensated the missing flow from the HPLC during waste positing operation. Automatic data acquisition was triggered using a short contact closure signal of the autosampler.

To gain higher selectivity, selected reaction monitoring (SRM) was chosen. Key parameter settings for SRM are given in Table 17.

Table 17: SRM data and retention time of macrolides, ionophores and tiamulin

	RT [min]	parent ion [amu]	daughter ion [amu]	collision energy [eV]
Oleandomycin	11.0	688.5	544.5	-20
Erythromycin	11.4	734.5	576.5	-22
Roxithromycin	12.9	837.5	679.5	-25
Internal standard	13.1	763.5	605.5	-24
Tiamulin	13.6	494.6	192.3	-27
Salinomycin	24.1	768.7	733.6	-22

2.2.1.8 Calibration

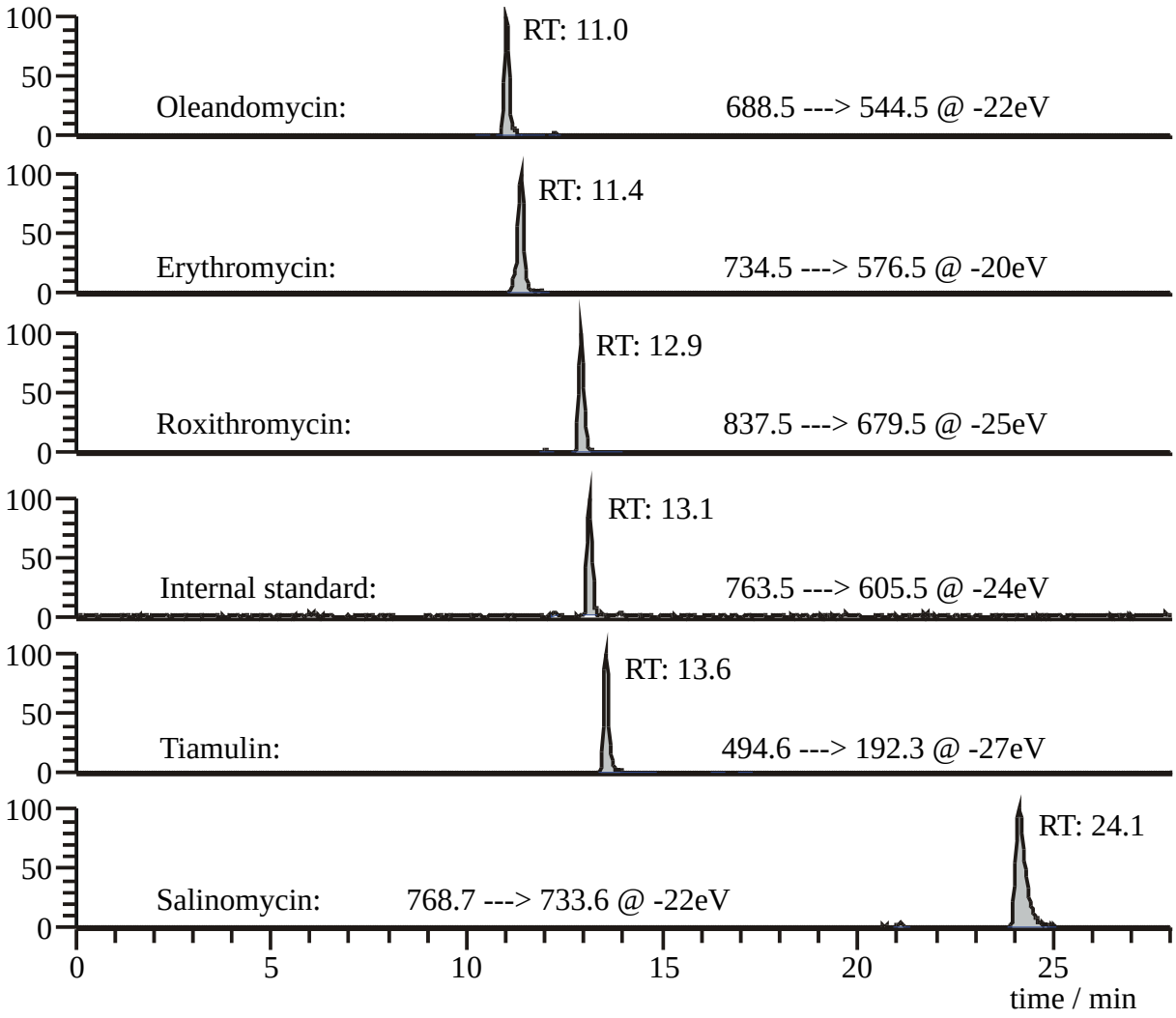
The calibration was performed as an internal standard calibration in the presence of manure matrix to account matrix effects [50, 51]. A liquid manure sample with a very high dissolved organic carbon (8.4 mg/mL) content and a relative high dry weight (11 %) was selected from an organic-pig farm to simulate a worst-case scenario. This antibiotic-free manure had a pH of 7.7. The cleaned-up extracts of this manure were used for preparation of the standards in the presence of manure matrix for HPLC-MS/MS determination.

A stock solution was produced by dissolving 10 mg of the macrolides, ionophores and tiamulin in 100 mL acetonitrile. This standard solution was stored at 4 °C in the dark and was stable for at least 3 months. Calibration standards (5, 10, 50, 100, 500, 1,000 and 5,000 ng/mL) were made by serial dilution of the stock solution. The internal standard solution was added to the calibration standards in a concentration of 500 ng/mL. The calibration standard solution (0.5 mL) was filled in 1.8-mL HPLC vials and 0.5 mL manure matrix were added. The manure matrix solution was produced by the established method described above. The calibration curves were calculated using a weighted (1/X) linear regression model.

2.2.2 Results and discussion to antibiotics in manure

All analytes were completely separated by HPLC. The selected APCI SRM traces for quantification are shown in Figure 4.

Figure 4: APCI SRM traces of selected macrolides, ionophores and tiamulin for quantification in spiked manure (600 µg/kg)



The calibration graphs are linear in the range from the limit of quantification (LOQ) up to 5,000 ng/mL with regression coefficients (R^2) better than 0.98 (Table 18).

Table 18: Calibration curve (with intercept and slope) and regression coefficient (R^2) of the weighted (1/X) matrix calibration with atmospheric pressure chemical ionisation in SRM mode

	intercept [Area ratio]	**slope [Area ratio/ng·ml^{-1}]**	**R^2**
Erythromycin	$-592.4 \cdot 10^{-5}$	$190 \cdot 10^{-5}$	0.993
Oleandomycin	$-276.3 \cdot 10^{-5}$	$273 \cdot 10^{-5}$	0.997
Roxithromycin	$-560.7 \cdot 10^{-5}$	$210 \cdot 10^{-5}$	0.998
Salinomycin	$-991.2 \cdot 10^{-5}$	$77.9 \cdot 10^{-5}$	0.991
Tiamulin	$-2466 \cdot 10^{-5}$	$975 \cdot 10^{-5}$	0.998

2.2.2.1 Validation of the method

The method was validated by spiking 15 g of homogenised antibiotic-free manure, as described above, with aliquots of 0.3-300 µl of the stock solution (2, 6, 20, 200 and 2,000 µg/kg manure) and shaking manually for 1 min. The following sample preparation, extraction and clean-up was identical to the procedures described above.

Recovery experiments for the macrolides, ionophores and tiamulin were carried out at five concentrations levels in triplicate.

The recoveries are given in Figure 5. Since there was no significant concentration (2, 6, 20, 200 and 2,000 µg/kg) dependency of the recovery rates, all results of all recovery experiments were averaged (Table 19).

Figure 5: Recovery rates of tiamulin and roxithromycin at five concentration levels (2, 6, 20, 200 and 2,000 µg/kg manure) The standard deviation (SD) for three replicates is indicated by an error bar, the standard deviation of the validated method is indicated by a dashed line.

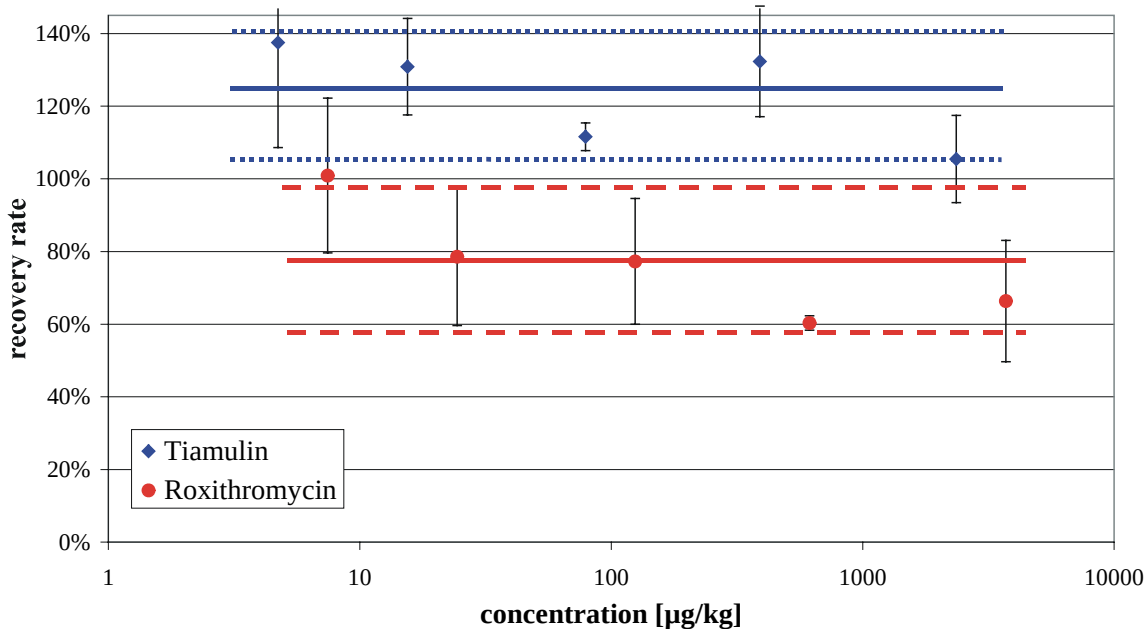


Table 19: Mean recovery, standard deviation (SD), relative standard deviation (RSD), limit of detection (LOD) and limit of quantification (LOQ), (three extractions, repetitions for each concentration level) of macrolides, ionophores and tiamulin in manure. Recoveries were determined at concentrations of 2, 6, 20, 200 and 2,000 µg/kg manure. LOD: S/N = 3:1, LOQ: S/N = 10:1

	Mean recovery	SD	RSD	LOD [µg/kg]	LOQ [µg/kg]
Erythromycin	94%	34%	36%	1.0	3.4
Oleandomycin	75%	16%	21%	0.4	1.4
Roxithromycin	78%	20%	15%	0.8	2.7
Salinomycin	119%	26%	22%	3.2	11
Tiamulin	123%	18%	15%	0.4	1.4
Ivermectin	n.v.	-	-	28	93
Monensin	n.v.	-	-	18	60
Tylosin	n.v.	-	-	20	68

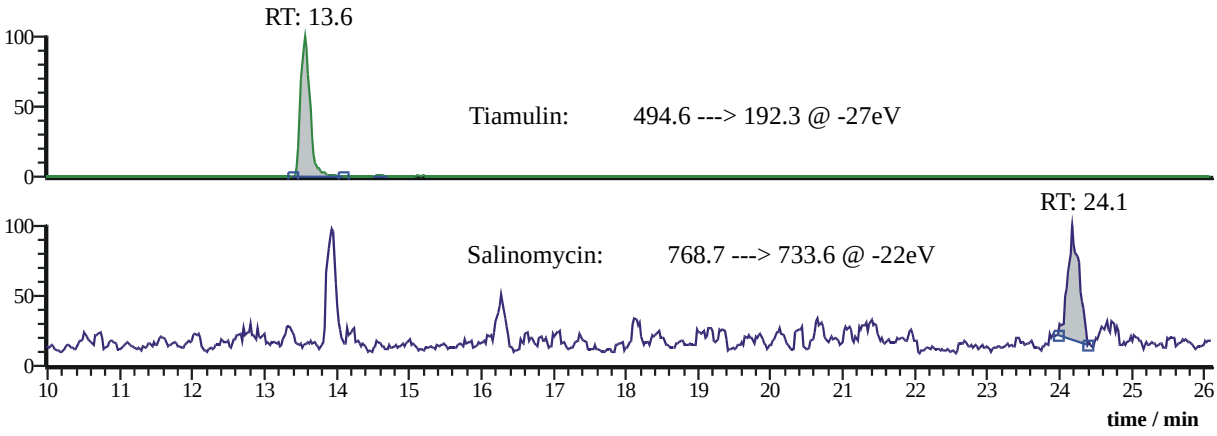
n.v.: not validated

Mean recoveries of 75% (RSD 21%) to 94% (RSD 36%) were obtained for the macrolides, the recovery of salinomycin was 119% (RSD 26%) and of tiamulin 123% (RSD 15%). The limit of detection (LOD) was taken as a signal-to-noise ratio of 3:1 and the limit of quantification (LOQ) was defined as a signal-to-noise ratio of 10:1 (Table 19). This method was also applied to ivermectin, monensin and tylosin, but did not give constant recovery rates for these three compounds.

2.2.2.2 Application to environmental samples

The method was tested for several samples in order to investigate the persistence of antibiotics in different manure samples. One of the four samples investigated contained tiamulin (43 µg/kg) and salinomycin (11 µg/kg). Figure 6 shows the SRM trace of the manure sample which contained tiamulin and salinomycin.

Figure 6: APCI SRM traces of a manure sample from a farm, that applied tiamulin and salinomycin. Tiamulin (43 µg/kg) and salinomycin (11 µg/kg) were measured.



Additionally the respective farmer gave the information that both compounds had been applied two months before sampling, together with information on dosage. The manure was stored several months, before it was homogenised in the manure tank and successively sampled. This manure had a dry weight of 5 % and the total organic carbon was 29 mg/mL. The concentrations of tiamulin and salinomycin were two orders of magnitude lower than the expected concentrations of 2,000 µg/kg manure [52, 53]. This expected concentration is based

on the assumption that the administered dosage of 2 kg antibiotic is excreted completely by the 800 pigs and the whole liquid manure was deposited in the 1,000 m^3 manure tank.
These antibiotics are probably not very stable in manure. Time and temperature-dependent degradation experiments are necessary to obtain more information about the long-term stability of these compounds in manure (see chapter 2.3).

2.3 Manure degradation experiment

2.3.1 Experimental to the manure degradation experiment

Chemicals, sample pre-treatment, liquid-liquid extraction, SPE clean-up, HPLC and mass spectrometry conditions were the same as described above (see chapter 2.2.1.)

2.3.1.1 Manure

Ten litre liquid manure were collected in May 2002 directly from the manure tank of a local farmer. The manure in the tank was stirred for about 20 minutes before sampling. The temperature of the liquid manure was about 18 °C.
There was no application of erythromycin, roxithromycin, tiamulin and salinomycin for about 8 months before sampling. The concentrations of these four antibiotics in the sampled manure were below the limit of detection.

2.3.1.2 Preparation of the degradation experiment

30 Erlenmeyer flasks were filled with 100 g fresh manure that was spiked with a mixture of antibiotics at a concentration of 2,000 µg/kg to mimic excretion of treated animals. This concentration is based on the assumption that an administered dosage of 2 kg antibiotic is excreted completely by 800 pigs and that all of the liquid manure is stored in a 1,000 m^3 manure tank, which is the amount used by the local farmers [46].
The Erlenmeyer flasks were stored in the dark at 20°C (Memmert, Modell 800, Schabach, Germany) and closed with a fermenting tube in order to maintain anaerobic conditions. The loss of water during the experiment was compensated weekly by addition of HPLC-grade water after difference weighting. After a specified time period, the samples were homogenised, extracted and analysed. Three sub-samples of 15 g homogenised manure each were analysed.

2.3.2 Results of the manure degradation experiment

2.3.2.1 Degradation experiment

The concentrations (c) of erythromycin, roxithromycin, salinomycin and tiamulin during the degradation experiment are displayed in Figure 7a - Figure 10a. To obtain detailed insight, the data are shown on a natural log scale in Figure 7b - Figure 10b. Each point is the average of three replicate extractions of a single incubation. From these data, kinetic data such as half-lives are calculated and presented in Table 20.

Table 20: Slope of the linear regression, regression coefficient (R^2) and half-life of macrolides, salinomycin and tiamulin during manure storage.

	slope (k)	**R^2**	**Half-life**
Erythromycin	-0.017	0.98	41±1 days
Roxithromycin	-0.005	0.95	130±10 days
Salinomycin	-0.135	0.97	5.1±0.3 days
Tiamulin	-	-	>>200 days

Erythromycin

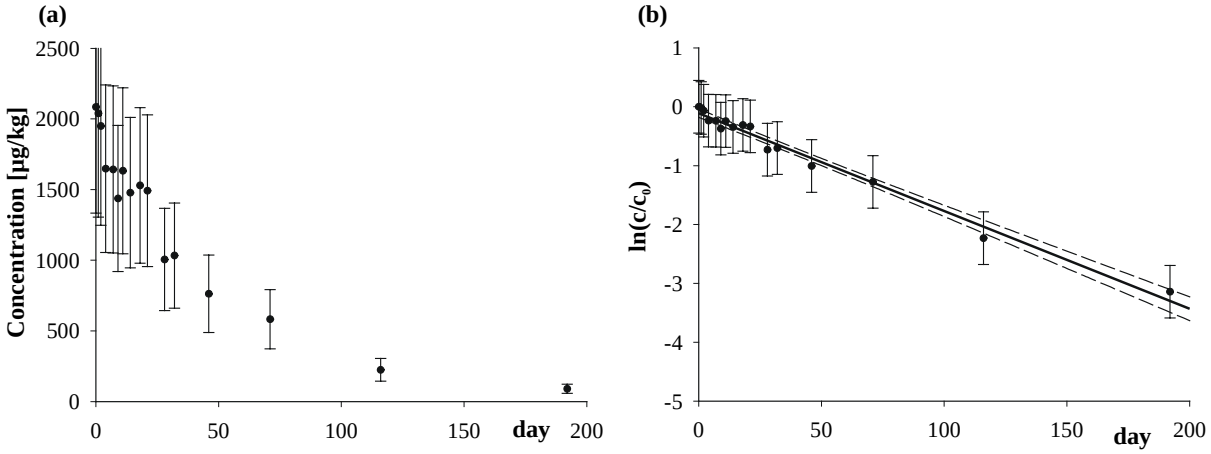
Erythromycin (Figure 7a) shows a typical first order degradation curve following equation (1) [54]:

$$c = c_0 \cdot e^{-k \cdot t} \qquad (1).$$

The natural logarithm of the concentration divided by the starting concentration (c_0) ($\ln c/c_0$) versus time (t) plot (Figure 7b) shows a straight line with a good regression (Table 20). Thus the degradation of erythromycin follows a first order degradation. From equation (2) a half-life of 41 days was calculated for erythromycin in manure.

$$t_{1/2} = \frac{\ln 2}{k} \qquad (2)$$

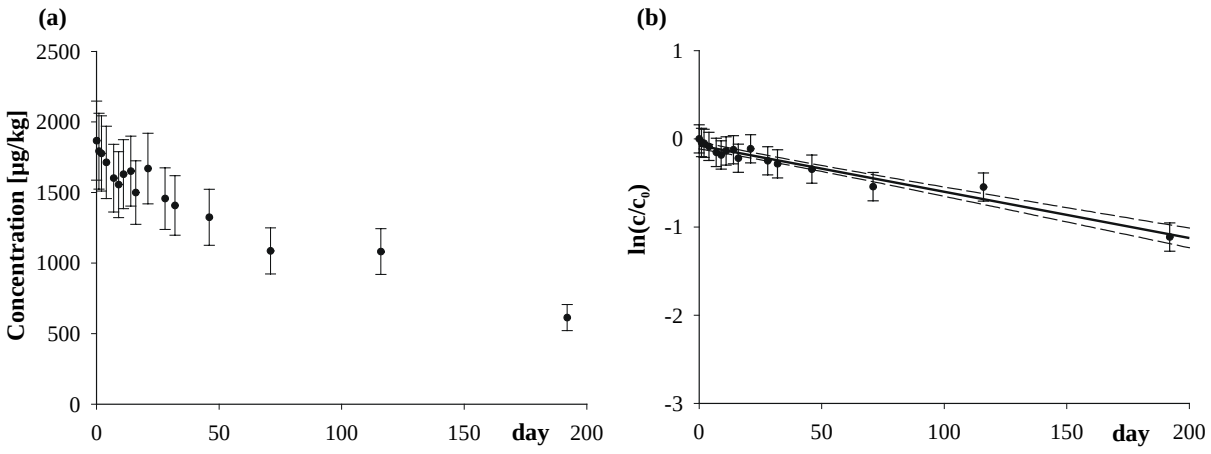
Figure 7: Concentration/time plot of erythromycin during an incubation of 16 single experiments in liquid manure (a) and plot of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of erythromycin (b) including the 95% confidence interval. Each point is the average of three extractions of one 100 g batch. Error bars are based on the standard deviation of the validated method.



Roxithromycin

The kinetics of roxithromycin (Figure 8a) was similar to those of erythromycin, but had a slower elimination rate. From the natural logarithm of the c/c_0 versus time plot (Figure 8b), a half-life of 130 days can be calculated by a kinetic expression.

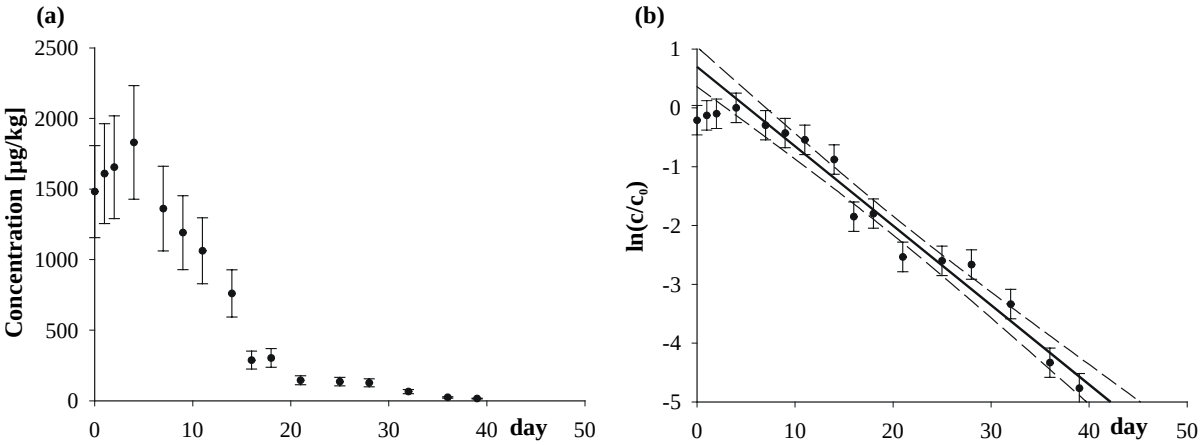
Figure 8: Concentration/time plot of roxithromycin during an incubation of 16 single experiments in liquid manure (a) and plot of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of roxithromycin (b) including the 95% confidence interval. Each point is the average of three extractions of one 100 g batch. Error bars are based on the standard deviation of the validated method.



Salinomycin

The degradation kinetics of salinomycin (Figure 9a) is different to those of the macrolides. The concentration of salinomycin remained constant for about four days before significant elimination started. If the elimination was due to biodegradation, the microorganisms responsible for degradation needed a lag-phase to adapt to salinomycin before they were able to metabolise this compound. After 40 days, the concentration of salinomycin was below the limit of quantification of the analytical method. A first order degradation and a half-life of 5 days could be calculated from the linear regression of the natural logarithm/time plot, starting from day four (Figure 9b).

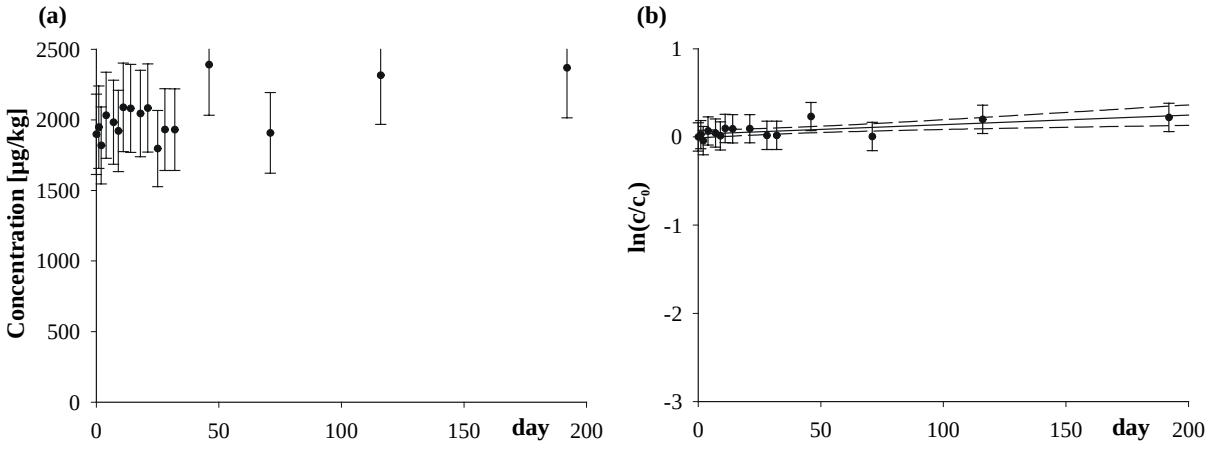
Figure 9: Concentration/time plot of salinomycin during an incubation of 16 single experiments in liquid manure (a) and plot of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of salinomycin (b) including the 95% confidence interval. Each point is the average of three extractions of one 100 g batch. Error bars are based on the standard deviation of the validated method.



Tiamulin

The same experiment with tiamulin (Figure 10) gave completely different results. The concentration of tiamulin remained constant during the course of the entire experiment. No degradation was detectable even after 180 days.

Figure 10: Concentration/time plot of tiamulin during an incubation of 16 single experiments in liquid manure (a) and plot of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of tiamulin (b) including the 95% confidence interval. Each point is the average of three extractions of one 100 g batch. Error bars are based on the standard deviation of the validated method.



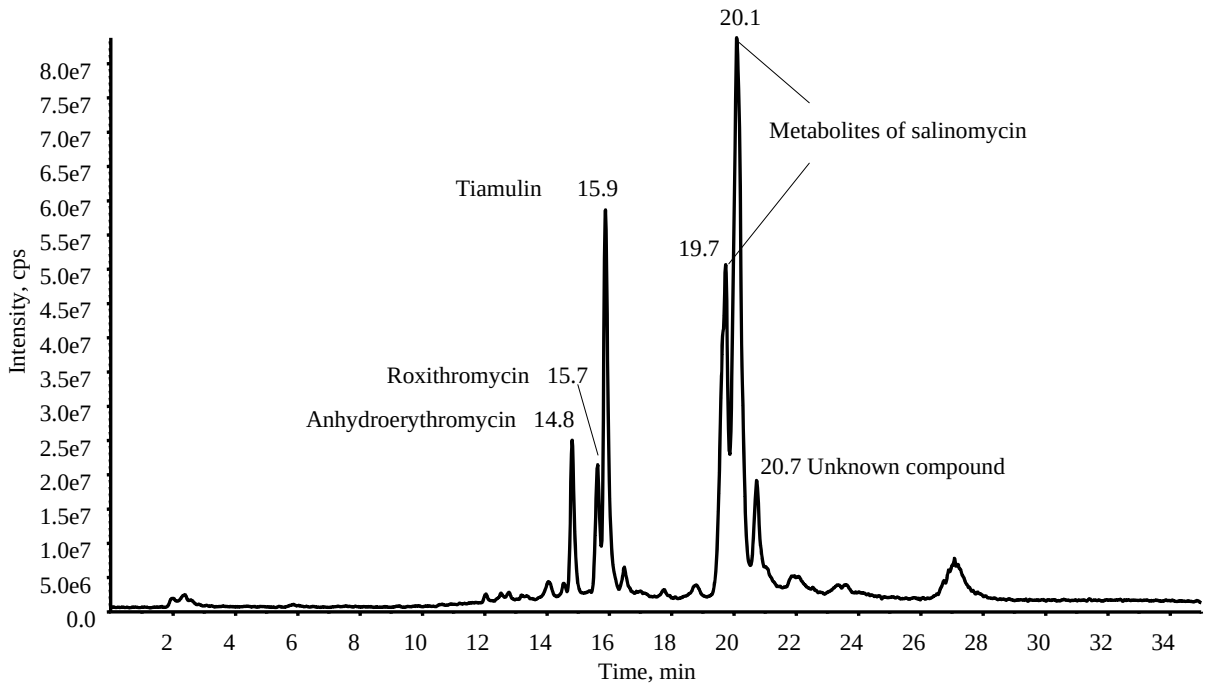
Tests for higher order kinetics were applied to all four degradation plots, but no correlation could be found. Thus it is assumed that the first order kinetics is applicable.

The standard deviation of the three replicates was in the same range or better as the standard deviation determined during the validation procedure for this analytical method. All point were within the 95% confidence intervals. The calculated half-lives and figures are based on the standard deviation of the validated method.

2.3.2.2 Determination of metabolites

The extract from day 192, the extract of blank manure and a standard solution were measured in a full scan HPLC-MS run in ESI positive mode with the same HPLC conditions described above. For the sake of improved sensivity these experiments were performed on an API 2000 (Applied Biosystems, Darmstadt). All chromatograms were compared with each other. Four new peaks were determined in the extract of day 192 (Figure 11) in comparison to the blank and standard solution. The same HPLC experiment with a high resolution mass spectrometer with electrospray ionisation in positive mode (Bio TOF III, Bruker, Bremen, Germany) resulted in high resolution mass spectras (HR-MS) of the chromatographic peaks at 19.7 min, 20.1 min and 20.7 min. By means of ISOFORM Version 1.02 (NIST, United States) and the HR-MS data the empirical formula of all three peaks could be identified (Table 21).

Figure 11: Full scan HPLC-ESI-MS run of the extract of day 192. Metabolites of salinomycin could be detected at 19.7 and 20.1 min and a metabolite of erythromycin at 14.8 min. Residues of tiamulin (15.9 min) and roxithromycin (15.7 min) and a unknown compound at 20.7 min.

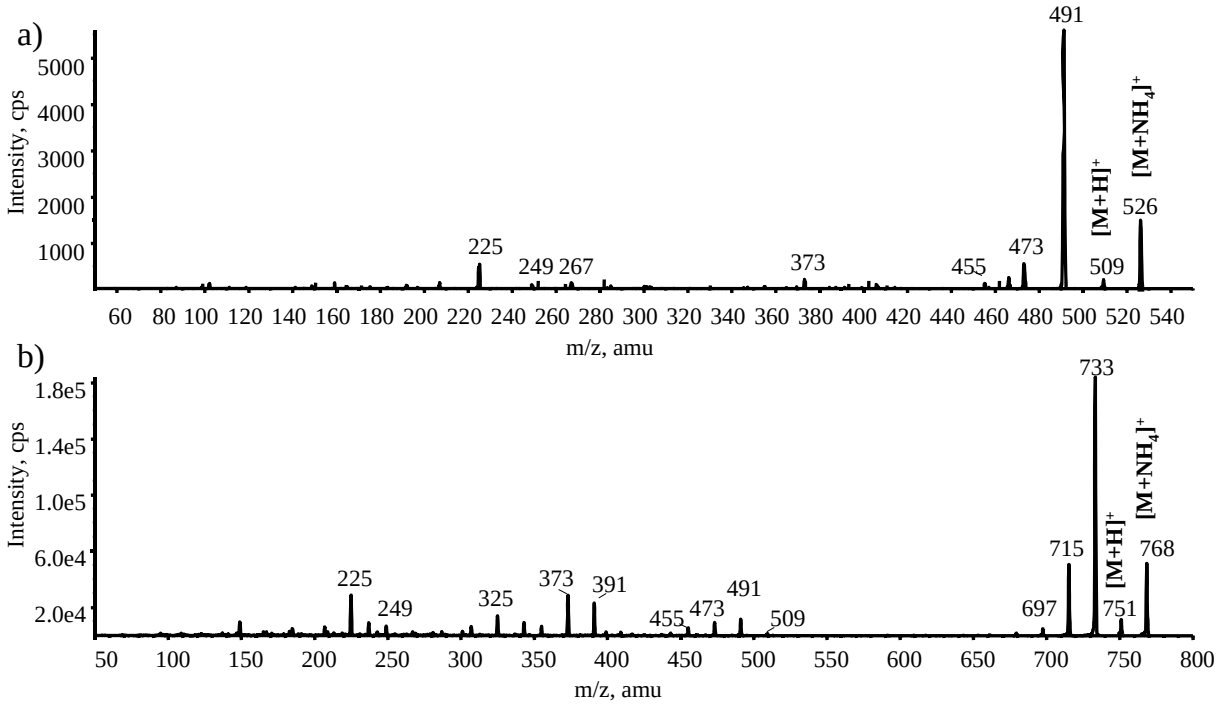


Metabolites of salinomycin

Peak 19.7 min and 20.1 min were identified as two isomers of $C_{29}H_{48}O_7$. The product ion scan (API 2000, Applied Biosystems, Darmstadt) of the ammonia adduct of $C_{29}H_{48}O_7$ (Figure 12a) showed a similar spectrum as the product ion scan of the ammonia adduct of salinomycin (Figure 12b). It seems that the new formed metabolite resulted by a cleavage of salinomycin. Vértesy *et al.* described in their work a microbial decomposition product of salinomycin with the same empirical formula [55].

The peak at 20.7 could be identified by HR-MS as $C_{29}H_{46}O_7$ (Table 21). The concentration of this metabolite was too small for further mass spectrometric experiments.

Figure 12: Product ion scan of the ammonia adduct of the metabolite of salinomycin, 526 (a) and product ion scan of the ammonia adduct of salinomycin, 768 (b)



Metabolites of erythromycin

By means of two MRM transitions, 716 --> 158 @ 45 eV, 716 --> 558 @ 25eV, and the same retention time of a standard solution, the peak at 14.8 was identified as anhydroerythromycin, a well known metabolite of erythromycin [56]. The synthesis of anhydroerythromcin has been described by McArdell *et al.* [57].

Table 21: Measured mass of the three peaks at 19.7 min, 20.1 min and 20.7 min of a high resolution fullscan HPLC-ESI-TOF-MS run. Suggested elemental composition, theoretical mass for the suggested ion of the suggested composition and difference of the mass of the suggested elemental composition and measured mass in ppm.

RT [min]	Measured mass [amu]	Suggested elemental composition	Theoretical mass for the suggested ion [amu]	difference [ppm]
19.7	509.3466	$[C_{29}H_{48}O_7+H]^+$	509.3473	1.4
	526.3726	$[C_{29}H_{48}O_7+NH_4]^+$	526.3744	3.4
	531.3286	$[C_{29}H_{48}O_7+Na]^+$	531.3292	1.1
20.1	509.3482	$[C_{29}H_{48}O_7+H]^+$	509.3473	1.8
	526.3729	$[C_{29}H_{48}O_7+NH_4]^+$	526.3744	2.8
	531.3293	$[C_{29}H_{48}O_7+Na]^+$	531.3292	0.2
20.7	524.3586	$[C_{29}H_{46}O_7+NH_4]^+$	524.3488	1.9
	529.3149	$[C_{29}H_{46}O_7+Na]^+$	529.3142	1.3

2.3.3 Discussion to the manure degradation experiment

Before manure is spread on fields, the manure is stored up to 180 days in tanks of the farmers in the region of North-Rhine-Westphalia, Germany.

Erythromycin is used mostly as a one-time application in the beginning of the fattening phase of pigs. A one-time application means one application over 10-20 days during the lifetime of a pig for the treatment of an infection. With a half-life of 41 days, the excreted erythromycin is degraded up to 95 % during a 180 day storage in manure tanks. If the application of erythromycin is stopped a considerable time before the manure is applied to the fields, the contamination of the soil with high concentrations of this antibiotic can be prevented. Since the metabolite of erythromycin, anhydroerythromycin, has no antibiotic activity [56] a risk for the environment for this metabolite is probably lower than for the parent compound.

Roxithromycin is currently not used as a veterinary antibiotic; however, it is used in human medicine. This leads to significant concentrations in wastewater [35]. This degradation

experiment with liquid manure may give some basic insight into the behaviour of roxithromycin in sewage treatment especially in the anaerobic processes in these plants, i.e. the digester.
As a result of an incomplete elimination of roxithromycin in the digester, roxithromycin can enter the environment if digested sludge is used as fertiliser on agricultural fields.

Salinomycin shows a rapid degradation with a half-life of 5 days in this manure; therefore, the application of salinomycin might be considered to be less problematic. However, salinomycin is used as a feed additive for the prevention of the coccidiosis and for growth promotion. Growth promoting is the continuous application of the antibiotic to promote weight gain in the fattening phase of pigs. In both cases, a continuous flow of freshly excreted salinomycin comes from the stables to the manure tank. A 99 % degradation of salinomycin in the manure tanks requires about 38 days. Thus there is not enough time for the complete degradation of salinomycin, the soil may be contaminated with salinomycin if this manure is dispersed on the fields. The newly formed metabolite of salinomycin has, according to Vértesy *et al.* [55], no antibiotic activity and is no longer capable to complexing sodium or potassium. However, there is no full risk evaluation for this compound yet.

In contrast, tiamulin showed no degradation. When manure containing tiamulin is spread on the fields, the soil will be contaminated. These conclusions correspond with other studies [58] where tiamulin and salinomycin were detected two months after the application in liquid manure at concentrations of 11 µg/kg and 43 µg/kg, respectively. Also, tiamulin was detected in soil which was fertilised with manure that contained tiamulin a considerable time before sampling [59].
Haller *et al.* [60] and Tolls [44] have demonstrated that the antibiotics tylosin, sulfonamides and tetracyclines are persistent in soil and manure as well. Gavalchin and Katz reported the half-life of chlortetracycline of above 30 days and tylosin of 5 days in a manure soil matrix under aerobic conditions at 20 °C [61]. Also Hamscher *et al.* and Pfeifer *et al.* found high concentrations of tetracyclines and sulfonamides in liquid manure several months after application to pigs [41, 51].
From the data presented here, it seems that the use of some macrolides and polyether antibiotics would be preferable to sulfonamides and tetracyclines, while tiamulin should be avoided, if environmental aspects are taken into account.

2.3.4 Conclusions to antibiotics in manure

A precise and rapid multimethod with low LOQ has been developed to analyse macrolides, ionophores and tiamulin in manure. Liquid-liquid extraction followed by a diol SPE clean-up step resulted in sufficient clean extracts, which were analysed by HPLC-APCI$^+$-MS/MS. Recoveries for the macrolides ranged from 75 – 94 %, for the ionophore salinomycin the recovery rate was 119 %, while the pleuromutilin tiamulin has a recovery rate of 123 %, i.e. salinomycin and tiamulin are not significantly higher than 100 %. Recoveries were not dependent on the concentration level. No blank problems were detected during the method validation and the applications. The limit of detection ranged from 0.4-3.3 µg/kg, and LOQs ranged from 1.4-11.0 µg/kg. In the tested samples tiamulin was found at concentrations of 43 µg/kg manure and salinomycin at concentrations of 11 µg/kg. This method is more sensitive that of Hamscher *et al.* who investigated tetracycline antibiotics in manure [41].
The degradation experiment showed that tiamulin, which is a pleuromutilin compound, is persistent in manure. Additionally, it was shown that macrolides, as well as the polyether antibiotic salinomycin, are degraded under the conditions prevalent in manure tanks. Metabolites of salinomycin and erythromycin were detected in this experiment and a new metabolite was found. However, some questions about the persistence of these metabolites remain, as well as more basic discussions on the usage of antibiotics in industrial agriculture.

As tiamulin is very persistent in manure, its use should be avoided if environmental issues are taken in consideration.

Further degradation experiments of other antibiotics such as sulfonamides and tetracyclines in liquid manure under anaerobic conditions are necessary to create guidelines for farmers that will give sufficient time between termination of antibiotic use in their livestock and manure spreading.

3 Occurrence and fate of antibiotics in soil

3.1 Introduction to antibiotics in soil

The continuation of the veterinary route of pharmaceutical in the environment (Figure 1, path B) is the sink of antibiotics in soil [41, 43, 58, 60]. Large fractions of veterinary used antibiotics are transferred to manure tanks after application. Antibiotics which are applied to humans are transferred through the sewers to sewage treatment plants after excretion. The antibiotics may adsorb to the sewage sludge or leave the treatment plant unchanged with the STP discharge water [34, 35]. Manure and sewage sludge are dispersed on the fields and the antibiotics may contaminate the soil and eventually the ground water [62, 63, 64, 65]. After fertilising the fields with manure or sewage sludge which contains antibiotics, these compounds may undergo several processes resulting in complete or partial elimination of the parent compound [66]. Three different degradation behaviours are discussed:

1) Complete elimination through mineralisation of the antibiotics.

2) Partial transformation and

3) Persistence of the compounds in the environment.

This study describes the decrease of the antibiotics with an aerobic degradation experiment in soil during a 120-day time period. A reliable method to determine the more recently used antibiotics with macrocyclic, polyether or pleuromutilin structures has not been described in the open literature yet. However, some tetracyclines have been analysed by Hamscher *et al.* [41, 67]. Before the degradation experiment could be performed, a method for the determination of these antibiotics in soil must be developed. As soil is a complex matrix, an exhaustive extraction is important to obtain high recovery rates and an efficient clean-up procedure is necessary to remove interfering matrix components.

Compounds of interest are the macrolide antibiotics erythromycin and oleandomycin, which are used in human and veterinary medicine, roxithromycin, which is used only for human applications, and tylosin, a veterinary drug. The pleuromutilin derivative tiamulin, a veterinary antibiotic and the ionophore salinomycin, which is used for growth promoting in animal husbandry are studied as well. In this study the term degradation is used to describe decrease of concentration of the respective parent compound.

3.2 Method development and validation to antibiotics in soil

3.2.1 Experimental to antibiotics in soil

3.2.1.1 Chemicals to antibiotics in soil

Acetonitrile (HPLC-S gradient grade) was purchased from Biosolv (Valkenswaard, Netherlands). Water (HPLC grade) was obtained from Mallinckrodt Baker (Griesheim, Germany). Methanol (suprasolv grade) and acetone (analytical grade) were obtained from Merck (Darmstadt, Germany).

Ammonium acetate, aqueous ammonia solution (25 %) and glacial acetic acid were of analytical grade and were purchased from Merck. Erythromycin, tylosintartrate and roxithromycin were provided by Sigma-Aldrich (Seelze, Germany). Salinomycin SV sodium salt 2.5-hydrate, oleandomycin phosphate dihydrate and tiamulin fumarate (Vetranal^{TM}) were obtained from Riedel-de Haën (Seelze, Germany). The synthesis of (*E*)-9-[O-(2-methyloxime)]-erythromycin is described by Schlüsener *et al.* [58] and described above in chapter 2.2.1.2.

3.2.1.2 Soil

A typical German sandy loam soil (Monheim, Laacher Hof, AXXa, provided by Bayer Crop Protection, Leverkusen, Germany), which fulfils the requirements for standard pesticide registration studies, was taken from the surface layer (0-10 cm) in areas with vegetation for the degradation experiments. It had not received any antibiotic applications for at least 7 years. The soil was stored after sampling for one year maximum in a greenhouse with grass on the surface area and watered in regular intervals before the start of this study. Table 22 shows the complete physicochemical properties of the used soil.

Table 22: Physicochemical properties of the soil, taken from Monkiedje *et al.* [68].

Texture analysis (USDA)	
clay (<2µm) (%)	5
silt (<50-2µm) (%)	23
sand (2000-50µm) (%)	72
pH (water, ratio 1:2.5)	7.20
pH (0.01 M $CaCl_2$, ratio 1:2.5)	6.75
$C_{org.}$ (%)	1.69
$N_{org.}$ (%)	0.09
P (mg P_2O_5/100 g dry weight)	57.0
cation exchange capacity (mequiv/100 g dry weight)	8.0
maximum water holding capacity	34.4
density (g/ml)	2.5

3.2.1.3 Accelerated Solvent Extraction (ASE)

Humid soil samples which were taken from fields were stored at –20 °C prior to extraction. After defrosting 30 g of sieved (2 mm) humid soil were transferred into a 33 mL ASE extraction cell. Soils from the degradation experiment and for the method validation were filled directly into the cells. The cell was subsequently filled with Ottawa sand (20-30 mesh, Fischer Scientific, Schwerte, Germany) to reduce the void volume. The cells were sealed at both ends with circular cellulose filters (REF NO.: 321432, Schleicher & Schüll, Dassel, Germany) and end caps were fitted.

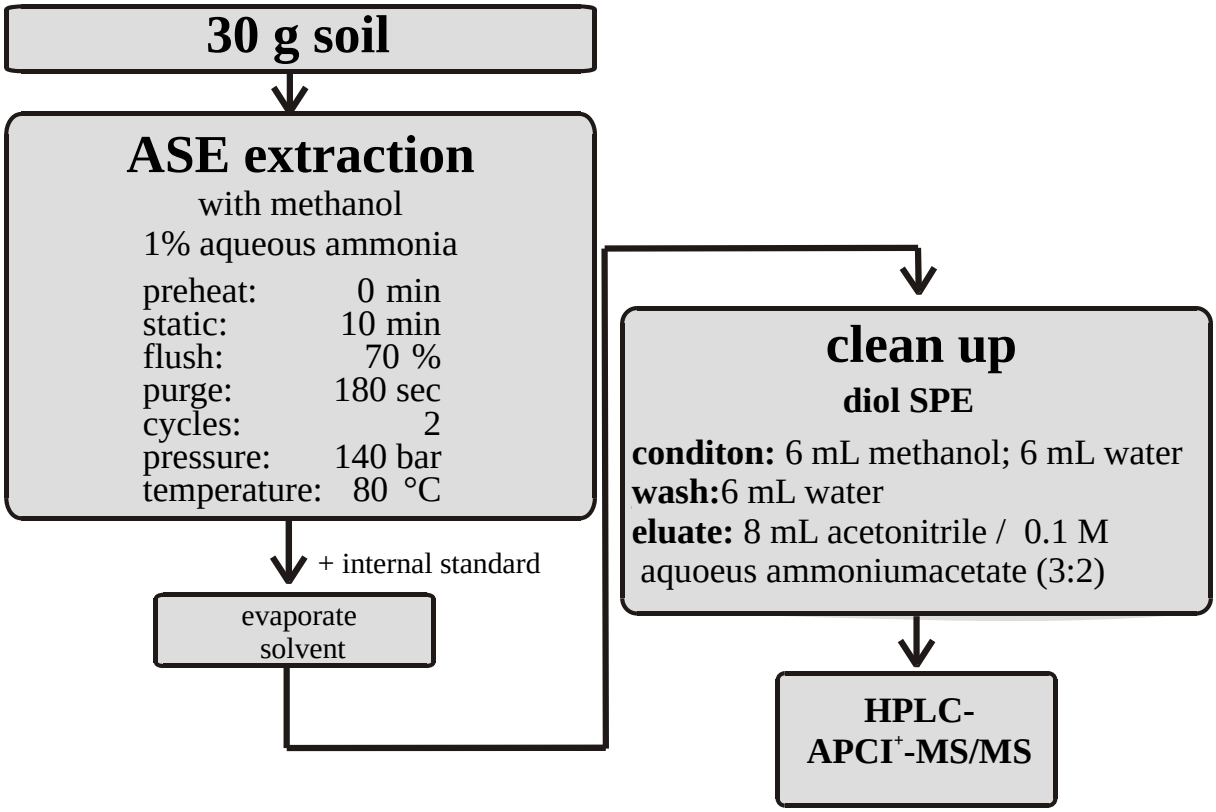
The ASE cells were extracted with aqueous ammonia in methanol (1% v/v) with following ASE conditions: preheat: 0 min; static: 10 min; flush: 70 %; purge: 180 sec; cycles: 2; pressure: 140 bar; temperature: 80 °C. After the extraction 25 µL of internal standard solution (10 mg (*E*)-9-[-O-(2-methyloxime)]- erythromycin in 100 mL acetonitrile), followed by 150 µL of glacial acetic acid were added to the extract and the flask was shaken by hand for 10 seconds. The mixture was poured in a 100 mL distilling flask and the sample volume was

reduced to 5 mL using a rotary evaporator at 60 °C and 290 mbar. The residue was dissolved in 15 mL water and the volume was reduced again to 10 mL at 60 °C and 150 mbar.

3.2.1.4 SPE clean-up to antibiotics in soil

Diol solid-phase extraction cartridges from UCT (2000 mg, Bristol PA, United States) were conditioned with 10 mL methanol followed by 10 mL water. A solid-phase extraction manifold (IST, Grenzach-Wyhlen, Germany) with PTFE stopcock and outlet was used. The soil extract (10 mL) was passed through the cartridge at a speed of 5 mL/min (vacuum). The cartridge was washed with 10 mL water to remove interfering matrix components, e.g. salts and co eluting matrix components. The SPE cartridges were eluted twice with 4 mL of an acetonitrile : 0.1 M aqueous ammonium acetate (3:2, v/v) mixture. An aliquot of 0.8 mL of the eluate was transferred to a 1.5-mL autosampler vial for HPLC-MS/MS analysis. Figure 13 shows the complete procedure for analysis of antibiotics in soil.

Figure 13: Sample preparation for soil



3.2.1.5 HPLC to antibiotics in soil

Separations were performed using a Phenosphere-Next RP18 column (2 mm i.d., length 150 mm, particle size 3 µm) and a SecurityGuard (Phenomenex, Torrance CA, United States) at 25 ± 1 °C. The flow rate was 0.2 mL/min. The HPLC gradient was established by mixing two mobile phases: phase A: 10 mM aqueous ammonium acetate solution and phase B: pure acetonitrile. Chromatographic separation was achieved with the following gradient: 0-1 min: 10% B, 1 min ->14 min: 10% -> 100% B, 14-29 min: 100% B, 29 min -> 30 min: 100% -> 10% B, 30-35 min: 10% B. Ten µL of each sample was injected.

The HPLC system consisted of a GINA 50 autosampler, a P 580A HPG HPLC pump, a degasser unit DEGASYS DG-1210 and a column oven STG 585 (all from Dionex, Idstein, Germany). The dead time of the HPLC system was 1.8 min. After HPLC separation, the analytes were determined by atmospheric pressure chemical ionisation / tandem mass spectrometry ($APCI^+$-MS/MS) in positive ion mode and selected reaction monitoring (SRM).

3.2.1.6 Mass spectrometry to antibiotics in soil

The triple quadrupole mass spectrometer (TSQ 7000, Finnigan-MAT, Bremen, Germany) was equipped with an APCI 2 source and operated under the following conditions: capillary temperature, 180 °C; sheath gas, 40 psi; corona current, 5 µA; vaporizer temperature, 450 °C; auxiliary gas, off; q_0 offset, -4.4 V; collision cell pressure, 2.0 mTorr; collision gas, argon; multiplier, 1900 V. The potential difference between the capillary and the tube lens was held at 70 V. The cycle time was 1.0 s during the chromatographic determination of the antibiotics. The data were processed using Xcalibur™ 1.3 software (Thermo Electron Corporation, West Palm Beach FL, United States). The silica capillary of the APCI 2 source was replaced by a steel capillary in order to reduce tailing of antibiotics adsorbing on the silica surface [49]. APCI was preferred because this ionisation is less vulnerable to matrix effects than ESI [50, 51].

A post-column Valco divert valve was used to direct most of the non-significant LC flow of a sample to waste. Diverting the flow minimised contamination of the MS source: 0-8 min divert to waste, 8-28 min flow to mass spectrometer, 28-35 min divert to waste. An additional flow of 50 µL/min water acetonitrile (3:7, v/v) pumped by a LC-10 AT HPLC (Shimadzu, Duisburg, Germany) compensated the missing flow from the HPLC during waste positing operation. Automatic data acquisition was triggered using a short contact closure signal of the autosampler.

To gain higher selectivity, selected reaction monitoring (SRM) was chosen. Key parameter settings for SRM are given in Table 23.

Table 23: Retention times as well as MS-conditions for the analysis of macrolides, salinomycin and tiamulin in soil

	Retention time	Precursor-Ion	Product-Ion	Collison-energy
Erythromycin	11.4 min	734.5	576.5	-22 eV
Roxithromycin	12.9 min	837.5	679.5	-25 eV
Salinomycin	24.1 min	768.7	733.6	-22 eV
Tiamulin	13.6 min	496.6	192.3	-27 eV
Oleandomycin	11.2 min	688.5	544.5	-20 eV
Tylosin	12.1 min	917.1	772.5	-32 eV

3.2.1.7 Calibration to antibiotics in soil

The calibration was performed as an internal standard calibration in the presence of soil matrix to overcome matrix effects [50, 51]. A typical German soil (Monheim, Laacher Hof, AXXa) was chosen for preparation of the standards in the presence of soil matrix for HPLC-MS/MS determination.

A stock solution was produced by dissolving 10 mg of the macrolides, ionophores and tiamulin in 100 mL acetonitrile. This standard solution was stored at 4 °C in the dark and was stable at least for 3 months. Calibration standards (5, 10, 50, 100, 500, 1,000 and 5,000 ng/mL) were made by serial dilution of the stock solution. The IS was added to the calibration standards in an amount of 500 ng/mL. The respective calibration standard solution (0.5 mL) was filled in 1.8-mL HPLC vials and 0.5 mL soil matrix was added. The soil matrix solution was produced by extracting ‘AXXa’ soil by ASE with successive SPE cleanup as described above. The calibration curves were calculated using a weighted (1/X) linear regression model.

3.2.1.8 Recovery experiments to antibiotics in soil

For validation of the method, 100 g of antibiotic-free soil from Laacher Hof were spiked with the stock solution (1; 6; 20; 200 and 2,000 µg/kg soil) using the following protocol:
To avoid potential effects of solvents upon the sorption of compounds to the soil, the volumes of the application solution (1-1,800 µL) were deposited evenly onto portions of ~10 g air-dry soil in porcelain dishes. Such treated samples of soils were thoroughly mixed with a spatula until the solvent was completely evaporated (~10 min) and the respective compounds were evenly distributed. The respective 10 g samples were subsequently added to the total soil mass of the corresponding soil (100 g). These 110 g soil samples were homogenised by means of a tumbling mixer for one hour. They were extracted within an hour after homogenisation. Control experiments after 24 h aging of the spiked soil gave the same recovery rates with this method.
Recovery experiments for the macrolides, ionophores and tiamulin were carried out at five concentration levels in triplicate.

3.2.2 Results and discussion to antibiotics in soil

All analytes were completely separated by HPLC. The calibration graphs show linearity in the range from the limit of quantitation (LOQ) up to 5,000 ng/mL with regression coefficients (R^2) better than 0.992.

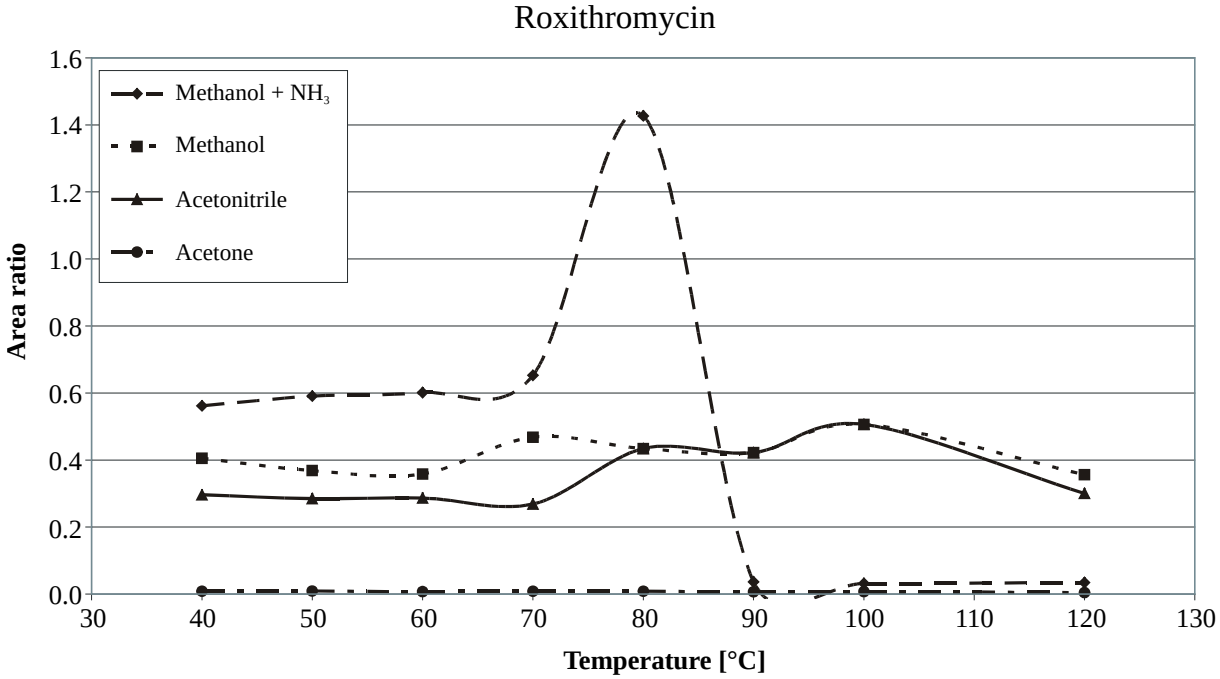
3.2.2.1 Results of temperature and solvent optimisation of the ASE

For the optimisation of the ASE conditions, samples of 10 g soil were each spiked with 50 µL stock solution (10 mg of macrolides, ionophores and tiamulin in 100 mL acetonitrile) and placed in an 11 mL ASE extraction cell. These samples were extracted three times into separate vials in four different experiments using acetone, acetonitrile, methanol and 1% (v/v) aqueous ammonia in methanol at temperatures varied from 40 to 120 °C in 10 ° steps (Fig. 3). These extracts were analysed directly after centrifugation (C-1200, NeoLab, Heidelberg, Germany) by HPLC-MS/MS. During this ASE optimisation experiment, all three extracts were analysed separately. Significant amounts of analytes were found in the second extract but no analytes were found in the third. In Figure 14 the results of the first and second extraction of roxithromycin as a function of temperature are summarised. The other analytes behave similarly. For all compounds, optimum extraction efficiency was found at 80°C and

140 bar. Above 80 °C the analytes may be hydrolysed. Temperature and pH value may help to dissolve the analytes from humic acids and other soil matrix compounds. Increasing the static extraction time from 10 min up to 20 min did not result in higher recovery rates. To get a more homogenous soil sample 33 mL extraction cells with 30 g sample were chosen for validation and the recovery study.

Figure 14: Extraction of roxithromycin in spiked soil samples with different solvents at various temperatures. The graph shows the relative extraction performance (Area ratio = Area $_{\text{roxithromycin}}$/Area $_{\text{internal standard}}$) depending on the temperature and the solvent.

This optimisation of the ASE conditions shows the best extraction performance with methanol: aqueous ammonia (1 % v/v) at 80 °C and 140 bar.

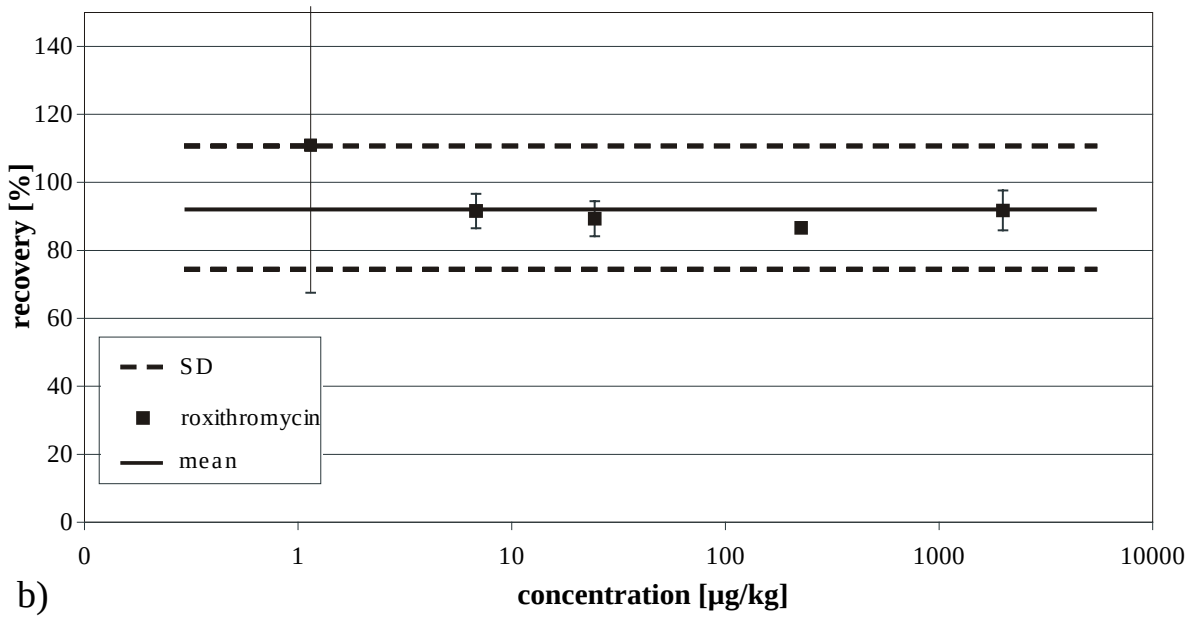


3.2.2.2 Results of the recovery study

The recovery rates of roxithromycin and tiamulin as selected samples are shown in Figure 15. Recovery rates of all experiments were averaged (Table 24) since there was no concentration (1, 6, 20, 200 and 2,000 µg/kg) dependency of recoveries. For the macrolides mean recoveries of 32% (RSD 23 %) to 93 % (RSD 20 %) were obtained. Salinomycin was found with 74 % (RSD 29 %) and tiamulin with 118 % (RSD 19 %). The limit of detection (LOD) was taken as a signal-to-noise ratio (S/N) of 3:1 and the limit of quantitation (LOQ) was defined as a signal-to-noise ratio of 10:1 (Table 24). No blank problems were detected during the method validation and the applications.

Figure 15: Recovery for roxithromycin (a) and tiamulin (b) at five concentration levels (1; 6; 20; 200 and 2,000 µg/kg soil). The standard deviation (SD) for three replicates is indicated by an error bar, the standard deviation of the validated method is indicated by a dashed line.

a)



b)

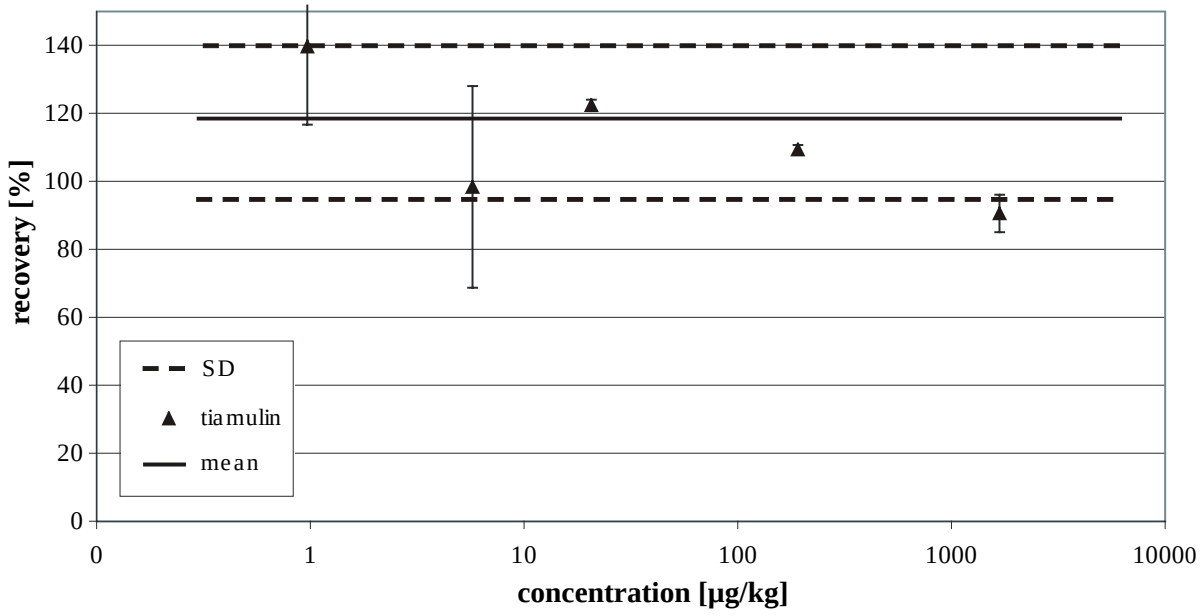


Table 24: Mean recovery, relative standard deviation (RSD) and limit of quantification (LOQ) (three extractions, repetitions for each concentration level) of macrolides, ionophores and tiamulin in soil. Recoveries were determined at concentrations of 1; 6; 20; 200 and 2,000 µg/kg manure. LOQ: S/N = 10:1

	Recovery rate [%]	RSD [%]	LOQ [µg/kg]
Erythromycin	43	23	1.4
Roxithromycin	94	19	1.0
Salinomycin	76	32	5.3
Tiamulin	118	18	0.6
Oleandomycin	38	51	1.4
Tylosin	32	23	30

The recovery rates of roxithromycin, tiamulin and salinomycin were in an acceptable analytical range. The recovery rates of oleandomycin, erythromycin and tylosin were generally too low, but no analytical method has been described in the open literature yet for soil with better results. The low recovery rates based on an sorption of the antibiotics to soil compounds. Especially tylosin builds complexes with calcium, which resulted in unextractable material. However, the recovery rates were sufficient enough for a degradation experiment in soil, because the recovery is constant over the working range of the degradation experiment.

3.2.2.3 Comparison to field data

The validated method was tested for several samples of two fields, in order to investigate the persistence of antibiotics in manure fertilised soil samples. Figure 16 shows the sampling procedure of two fields. Field I was only fertilised in February 2001 and field II was fertilised in August 2001 and February 2002 with liquid manure, which contained tiamulin (43 µg/kg) and salinomycin (11 µg/kg) [58]. Additionally the amount of applied manure was given (20 m^3/ha).

The samples were taken in November 2001 (white spots with Arabic numbers 1-12) and in May 2002 (yellow spots with letters A-I) from the first 30 cm of the surface layer. Additionally sample C and D were divided into two portions of 0-15 cm and 15-30 cm. The samples were stored at -20 °C prior extraction. Table 25 illustrates the concentration of tiamulin in these soil samples. Neither salinomycin, nor any other of the analysed antibiotics

were detected, though salinomycin was applied at the respective farm. Figure 17 shows the SRM trace of a soil sample extract, which contained tiamulin. The concentration ranged from 0.9 µg/kg up to 1.9 µg/kg soil.

From these data it can be shown that tiamulin persists in the soil. Even one year after fertilising the field with contaminated manure the concentration of tiamulin remains constant (Field I). The upper surface layer (0-15 cm) is more contaminated with tiamulin and the concentration was raising when the fields were fertilised again and again (Field II) with manure which contains tiamulin.

Figure 16: Sketch of the sampling of two fields. Samples were taken in November 2001 (spots 1-12) and in May 2002 (spots A-I) from the first 30 cm of the surface layer.

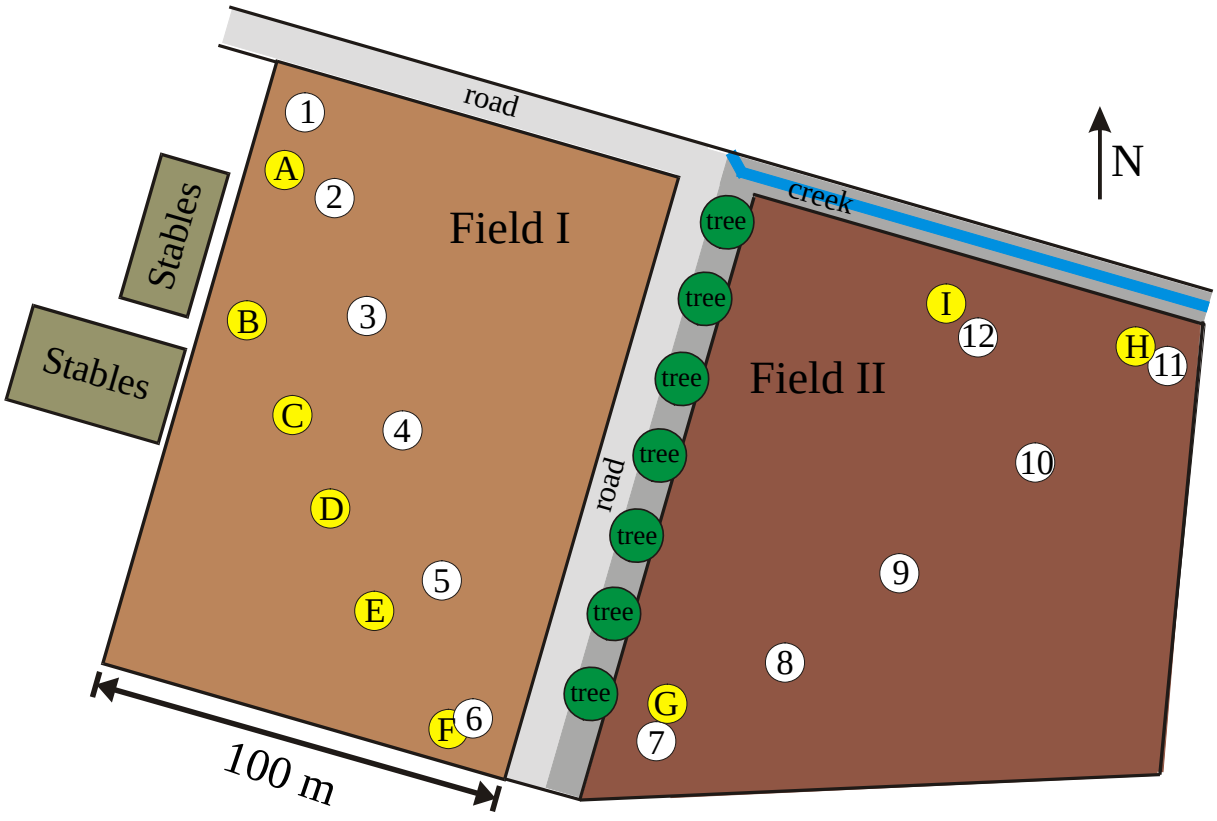


Figure 17: APCI SRM trace of a soil sample which contains tiamulin (0.9 µg/kg). The soil was fertilised with manure, which contained tiamulin (43 µg/kg), 9 months before sampling.

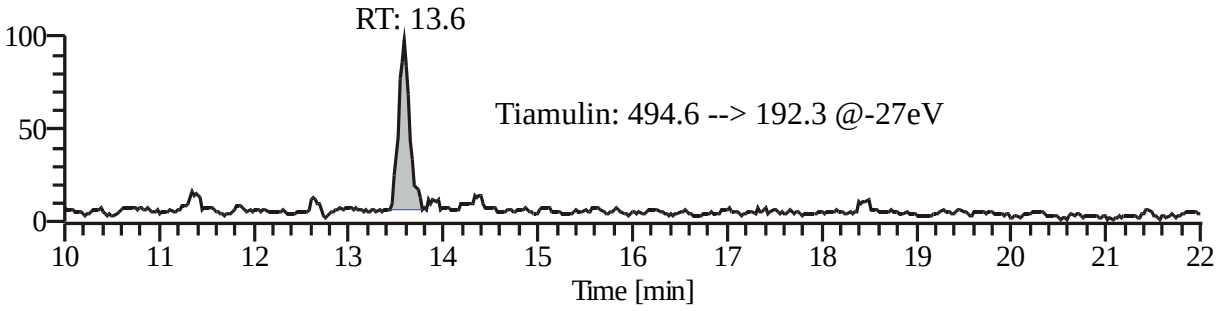


Table 25: Concentrations of tiamulin in soil. Field I was fertilised in February 2001 and field II was fertilised with liquid manure, which contained 43 µg/kg tiamulin, in August 2001 and February 2002.

	November 2001		May 2002	
	Sampling point	**Tiamulin [µg/kg]**	**Sampling point**	**Tiamulin [µg/kg]**
Field I	**(1)**	1.9	**(A)**	2.2
	(2)	1.2	**(B)**	1.5
	(3)	1.0	**(C, 0-15 cm)**	1.1
			(C, 15-30 cm)	0.5*
	(4)	1.4	**(D, 0-15 cm)**	1.1
			(D, 15-30cm)	0.6*
	(5)	1.4	**(E)**	1.3
	(6)	0.5	**(F)**	1.7
	Mean	**1.2**	**Mean**	**1.7**
Field II	**(7)**	0.9	**(G)**	1.1
	(8)	0.5*	**(H)**	1.1
	(9)	0.5*		
	(10)	0.5*		
	(11)	0.4*		
	(12)	<LOD	**(I)**	3.3
	Mean	**0.5**	**Mean**	**1.4**

*:<LOQ

From theoretical considerations a concentration of tiamulin in soil was predicted:

The PEC_{soil} was calculated with the following assumptions and formula (3):

density manure (ρ_{manure}) : 1,000 kg/m^3

concentration of antibiotics in the manure ($b_{antibiotic}$): 43 µg/kg [58]

area based load of manure (Q_N): 20 m^3/ha

density soil(ρ_{soil}): 1,800 kg/m^3

penetration depth of the antibiotics in soil (H_{depth}): 15 cm

$$PEC_{soil} = \frac{\rho_{manure} \cdot b_{antibiotic} \cdot Q_N}{\rho_{soil} \cdot 10000\,m^2 \cdot H_{depth}} \quad (3)\ [58]$$

The concentration of tiamulin (0.9 µg/kg) was higher than the predicted environmental concentration (PEC_{soil}) of 0.3 µg/kg soil [53]. This is different to Kümmerer's own improved data of (PEC_{soil}) 3-180 µg/kg soil [69]. Thus the concentrations in the field are slightly higher than predicted by Monforts, but considerably lower than predicted by Kümmerer [53, 69]. Possibly the concentration of tiamulin in the applied manure was higher than 43 µg/kg. Further experiments, like time- and temperature-dependent degradation experiments are necessary to obtain more information about the long-term stability of these compounds in soil.

3.3 Soil degradation experiment

3.3.1 Experimental to the soil degradation experiment

Chemicals, sample pre-treatment, ASE extraction, SPE clean-up, HPLC and mass spectrometry conditions were the same as described above. The same soil for the degradation experiment as for method development and matrix calibration was used (Table 22).

3.3.1.1 Degradation experiment

20 Erlenmeyer flasks were filled with 110 g sieved (2 mm) humid soil that was spiked with a mixture of antibiotics at a concentration of 2,000 µg/kg using the following protocol:
To avoid potential effects of solvents (acetonitrile) upon the sorption of compounds to the soil, the volumes of the application solution (1,800 µL) were deposited evenly onto portions of ~10 g air-dry soil in porcelain dishes. The thus treated samples of soils were thoroughly mixed with a spatula until the solvent was completely evaporated (~10 min) and the respective compounds were evenly distributed. The respective 10 g samples were

subsequently added to the total soil mass of the corresponding soil (100 g). These 110 g soil samples were homogenised by means of a tumbling mixer for one hour.

The Erlenmeyer flasks were stored in the dark at 20°C (Memmert, Modell 800, Schabach, Germany) and stoppered with glass wool to reduce water evaporation. The soil water content was about 12% (w/w). The loss of water during the experiment was compensated weekly with HPLC-water (Mallinckrodt Baker, Griesheim, Germany) by difference weighting. At 15 sampling times single flasks were analysed (t = 0, 1, 3, 5, 7, 12, 15, 19, 22, 28, 34, 42, 62, 85, 115 days).

Before the extraction started, the soil was mixed by shaking the Erlenmeyer flask for 1 min by hand. 30 g of soil were transferred into a 33 mL ASE (ASE 200, Dionex, Idstein, Germany) extraction cell. Three sub-samples of 30 g mixed soil from each sampling day were analysed.

3.3.2 Results and discussion to the soil degradation experiment

The concentrations (c) of erythromycin, roxithromycin, salinomycin, tiamulin, oleandomycin and tylosin during the degradation experiment are displayed in Figure 18a - Figure 23a. To obtain detailed insight, the data are shown on a natural log scale in Figure 18b - Figure 23b. Each point is the mean of three replicate extractions of a single incubation. From these data, kinetic data such as half-lives are calculated and presented in Table 26. Tests for higher order kinetics as first order were applied to all degradation plots, but no correlation could be fitted.

The standard deviation of the three replicates was in the same range or better as the standard deviation determined during the validation procedure for this analytical method. The calculated half-lives and figures are based on the standard deviation of the validated method.

Table 26: Slope of the linear regression, regression coefficient (R^2), half-life of macrolides, salinomycin and tiamulin

	slope (k)	R^2	Half-life
Erythromycin	-0.035	0.96	20±1.2 days
Roxithromycin	-	-	>>120 days
Oleandomycin	-0.031	0.91	27±2.5 days
Tylosin	-0.086	0.98	8.3±0.4 days
Salinomycin	-0.133	0.95	5.0±0.5 days
Tiamulin	-0.027	0.98	16±0.8 days

3.3.2.1 Erythromycin

Erythromycin (Figure 18) shows a typical first order degradation curve following equation (1) [54].

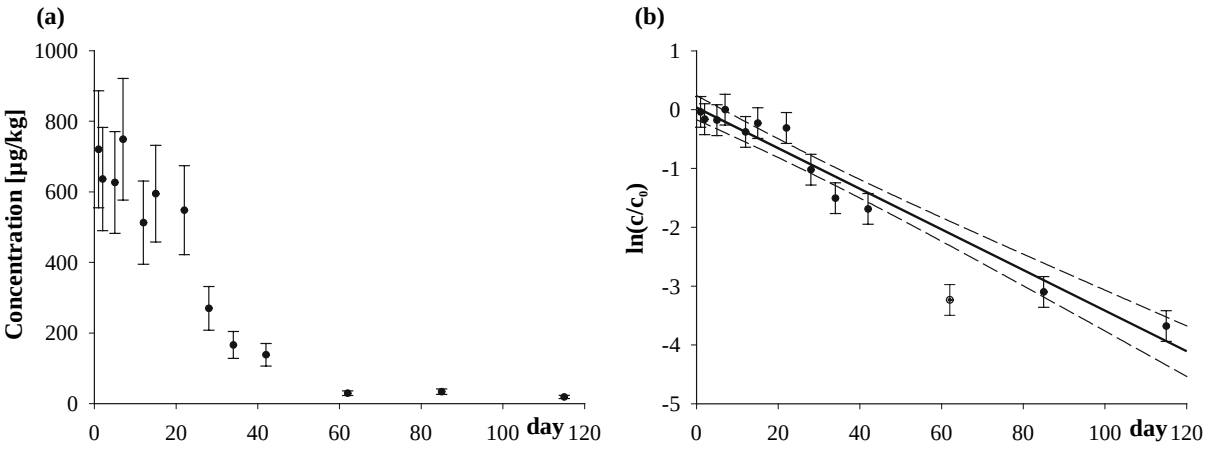
$$c = c_0 \cdot e^{-k \cdot t} \quad (1)$$

The natural logarithm of the concentration divided by the starting concentration (c_0) (ln c/c_0) versus time (t) plot shows a straight line with a regression coefficient of 0.96 (Table 26). All data points are within the 95 % confidence interval except day 62. This point was considerd to be an outlier and it was excluded for the calculation of the half-life. Thus the degradation of erythromycin follows a first order degradation. From equation (2) a half-life of 20 days was calculated for erythromycin in soil.

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

The result of this degradation experiment corresponds with results of Gavalchin and Katz [61], who investigated the degradation of erythromycin in a soil-faeces matrix via bioassay. This experiment does not have any implications for agriculture, but it is the only experiment, reported in the literature that is at least similar. These authors calculated a half-life of 12 days for this compound at 20 °C.

Figure 18: Concentration/time plot of erythromycin during an incubation of 15 single experiments in soil during an incubation of 15 single experiments in soil (a) and plots of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of erythromycin including the 95 % confidence interval. Each point is the average of three extractions of one 110 g batch.

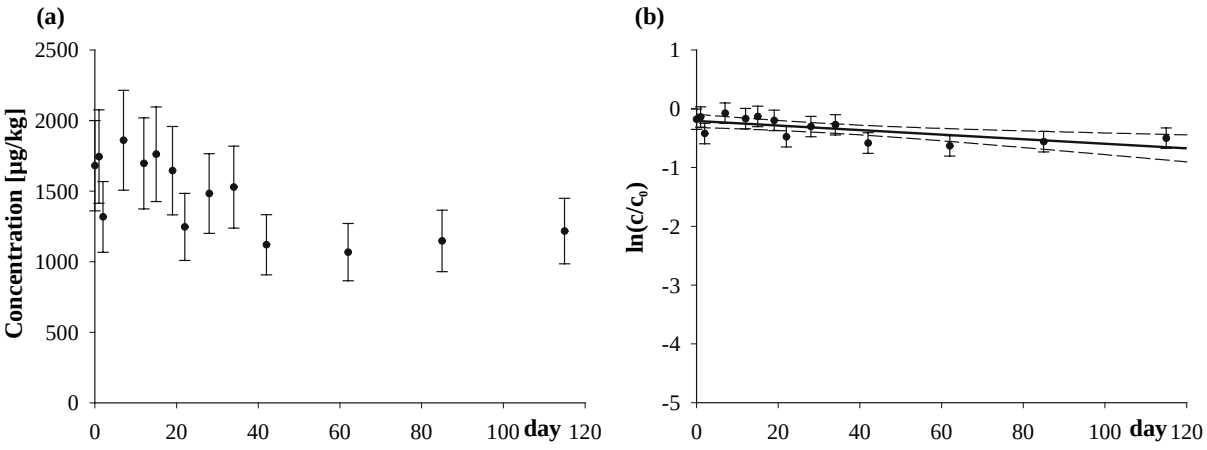


3.3.2.2 Roxithromycin

The same experiment with roxithromycin (Figure 19) produced completely different results. Only a slight decrease of the concentration was detectable even after 120 days. It was not possible to calculate a half-life.

If soil is fertilised with sewage sludge which may contain this antibiotic, the soil will be contaminated with roxithromycin and the compound may persist in the environment.

Figure 19: Concentration/time plot of roxithromycin during an incubation of 15 single experiments in soil during an incubation of 15 single experiments in soil (a) and plots of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of roxithromycin including the 95 % confidence interval. Each point is the average of three extractions of one 110 g batch

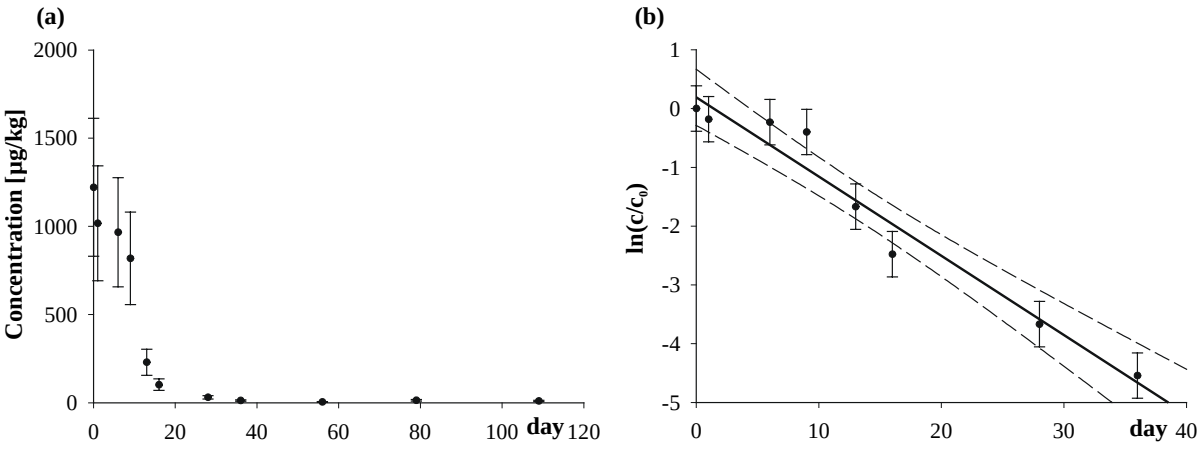


3.3.2.3 Salinomycin

The degradation of salinomycin (Figure 20) is faster than the degradation of erythromycin. After day 36, the concentration of salinomycin was below the limit of quantification of the analytical method. A first order degradation and half-life of 5 days was calculated from the linear regression of the natural logarithm/time plot.

With this short half-life in soil salinomycin should cause less problems in soil than sulfonamides and tetracyclines. However, an effect on soil microorganisms cannot be excluded.

Figure 20: Concentration/time plot of salinomycin during an incubation of 15 single experiments in soil during an incubation of 15 single experiments in soil (a) and plots of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of salinomycin including the 95 % confidence interval. Each point is the average of three extractions of one 110 g batch



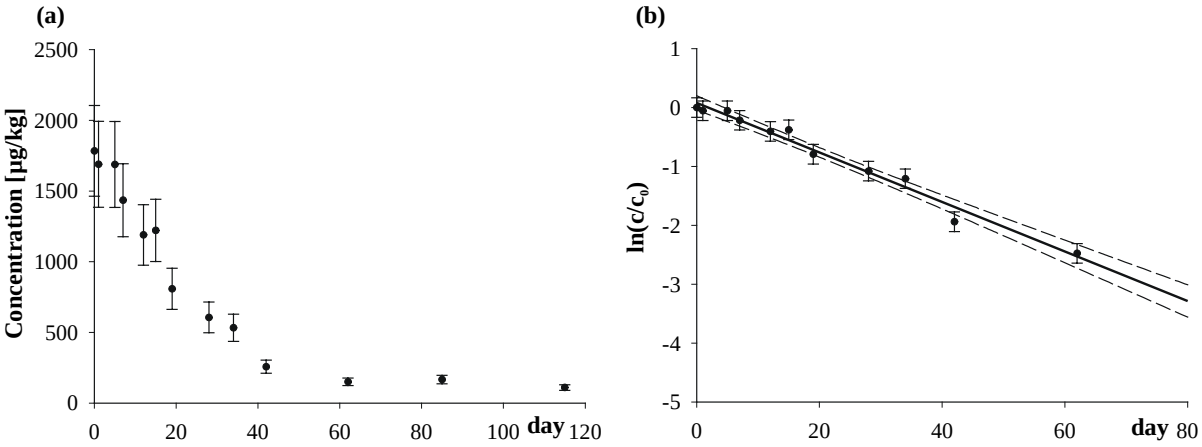
3.3.2.4 Tiamulin

Tiamulin (Figure 21) shows a typical first order degradation curve until day 62. After this day the concentration of tiamulin in soil seems to be constant. The natural logarithm of the concentration (c) divided by the starting concentration (c_0) (ln c/c_0) versus time (t) plot shows a straight line from day 0 to day 62. Therefore the degradation of tiamulin follows a first order degradation up to day 62. A half-life of 16 days was calculated for tiamulin in soil.

Further experiments have shown that tiamulin is present in liquid manure when the farmer used tiamulin to treat or prevent infections in their livestock [58]. After storing manure for about 180 days in manure tanks, tiamulin is still present in manure and shows no degradation [70]. The soil will be contaminated with tiamulin if this manure is used as fertiliser.

With a half-life of 16 days till day 62 and no degradation afterwards tiamulin persist for a long time in small concentrations after fertilising the field. This is in accordance with results from field sampling, Table 25 and [59].

Figure 21: Concentration/time plot of tiamulin during an incubation of 15 single experiments in soil during an incubation of 15 single experiments in soil (a) and plots of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of tiamulin including the 95 % confidence interval. Each point is the average of three extractions of one 110 g batch

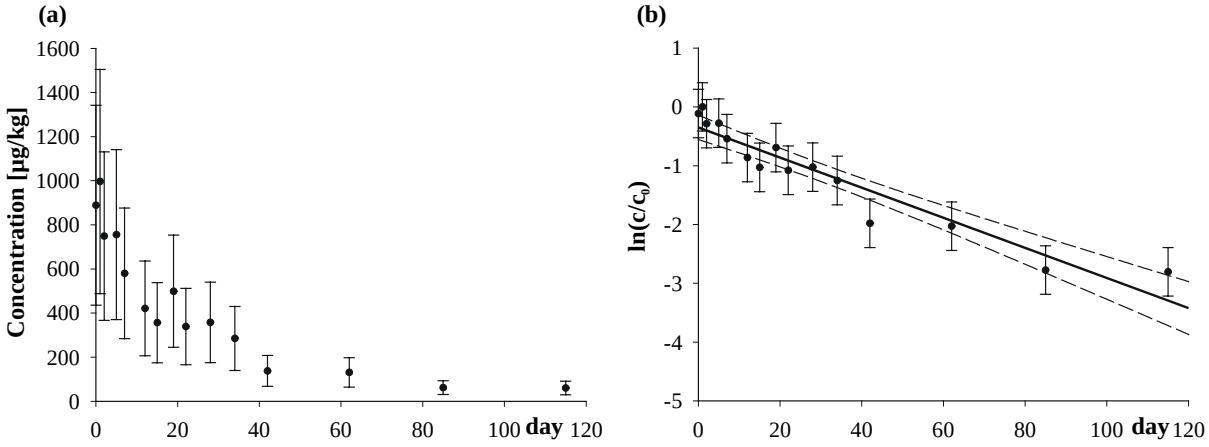


3.3.2.5 Oleandomycin

Oleandomycin (Figure 22) shows a typical first order degradation curve. A half-life of 27 days was calculated for oleandomycin from natural logarithm of the concentration divided by the starting concentration (c_0) (ln c/c_0) versus time (t) plot.

This antibiotic is more stable in soil than tylosin or erythromycin.

Figure 22: Concentration/time plot of oleandomycin during an incubation of 15 single experiments in soil during an incubation of 15 single experiments in soil (a) and plots of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of oleandomycin including the 95 % confidence interval. Each point is the average of three extractions of one 110 g batch

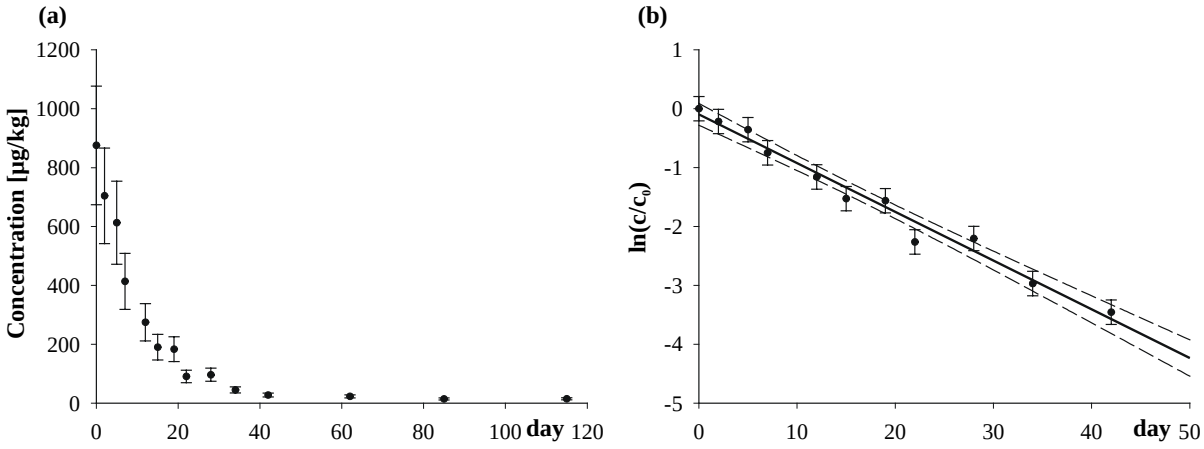


3.3.2.6 Tylosin

Tylosin (Figure 23) shows typical first order degradation curve. A half-life of 8 days was calculated for tylosin from natural logarithm of the concentration divided by the starting concentration (c_0) (ln c/c_0) versus time (t) plot.

Also Gavalchin and Katz found a rapid degradation of tylosin in a soil-faeces matrix at 20 °C. A half-life of 4-8 days was determined by Ingerslev *et al.* for tylosin in a soil-manure slurry [71].These experiment does not have any implications for agriculture, but these are the only experiments, reported in the literature that are at least similar.

Figure 23: Concentration/time plot of tylosin during an incubation of 15 single experiments in soil during an incubation of 15 single experiments in soil (a) and plots of the natural logarithm of the concentration/starting concentration (c/c_0) versus time of tylosin including the 95 % confidence interval. Each point is the average of three extractions of one 110 g batch.



3.3.3 Conclusions to antibiotics in soil

In the past, it has been demonstrated that antibiotics such as tylosin, sulfonamides, virginiamycin and tetracyclines are persistent in soil [41, 72, 73]. In this study it was demonstrated that indeed roxithromycin, which is a semi synthetic macrolide, is persistent in soil as well, while tiamulin is persistent at low concentrations.

Additionally it was demonstrated that erythromycin, oleandomycin, tylosin as well as the polyether antibiotic salinomycin, are degraded under conditions prevalent on fields. However, some questions about the persistence of metabolites remain. From the data presented in this study, it seems that the application of tylosin, erythromycin and polyether antibiotics might have less impact on soil than sulfonamides and tetracyclines. As roxithromycin is very persistent, fertilising the soil with sewage sludge should be avoided if environmental issues are taken in consideration. However, the longer a given antibiotic persists in the soil in an active form, the greater the potential for the bacterial populations of the soil to be affected, especially to become resistant. Effects on ground water or surface runoff water can not be excluded.

Also a rugged and rapid multiresidue method with low LOQ has been developed to analyse macrolides, ionophores and tiamulin in soil. ASE extraction followed by a diol SPE clean-up step resulted in sufficient clean extracts, which were analysed by HPLC-APCI$^+$-MS/MS. The LOQs that were obtained in this study are lower than those published by Hamscher *et al.* [41] who investigated tetracycline antibiotics in manure and soil.

4 Occurrence and fate of hormones and antibiotics in wastewater.

4.1 Determination and method validation of steroid hormones, hormone conjugates and macrolide antibiotics in influents and effluents of sewage treatment plants.

4.1.1 Introduction to the method validation of hormones and antibiotics in wastewater

The occurrence of endocrine disrupting chemicals as well as antibiotics in the environment has become an important issue in the last decades [74]. Especially steroid hormones and contraceptives are of special concern due to their endocrine potency [75]. The natural sex hormone estradiol, which has a high endocrine potential, its metabolites (estrone and estriol) and conjugates (glucuronides and sulfates) are mainly excreted by mammals [56]. Also the synthetic contraceptives ethinylestradiol and mestranol have high a endocrine potential and are excreted by women medicated by these drugs. Feminisation of fish living near the effluents of sewage treatment plants has been observed [76, 77]. Estrogenic effects on fish have been observed down to 1 ng/L in laboratory studies [78].

Antibiotics are also excreted by medicated humans and they have the potential to build resistant bacteria stems. Neu reported 1992 the resistance of bacteria to the majority of existing antibiotics [10]. These compounds are transferred through the sewers to sewage treatment plants and enter the environment through the effluent of the respective plants. Also an inhibition of the aerobic growth and inhibition of the nitrification of bateria in sewage sludge by antibacterial agents has been reported by Halling-Sørensen [79].

Several methods have been described in the literature to quantify steroid hormones in STP effluents. They are mostly based on solid phase extraction, derivatisation of the analytes and detection by GC-MS [80, 81]. The limits of quantification (LOQ) for such methods are in the range of low nanograms-per-liter. The determination of the estrogens in unfiltered STP influents needs a sufficient clean-up step to remove interfering matrix components [82].

Hormone-conjugates cannot be determined directly by GC-MS. These analytes must be enzymatically decomposed to gain the free estrogens prior to derivatisation and successive analysis by GC-MS [83 ,84 ,85]. On the other hand the conjugates can be determined directly by using liquid chromatography coupled with tandem mass spectrometry instead of GC-MS.

A method for the determination of roxithromycin, and clarithromycin as well as anhydroerythromycin, a transformation product of erythromycin without antibiotic activity [56], in effluents of sewage treatment plants has been reported by Göbel *et al.* and Hirsch *et al.* [15, 35]. In these studies erythromycin was transformed to anhydroerythromycin by using acidic conditions, thus it remains unclear whether the samples contained the parent or the transformation product.

The aim of this study was to develop a reproducible, robust and sensitive multiresidue method based on the recommendation of Green [86] to investigate the fate of estrone (E1), 17β-estradiol (E2), estriol (E3), 16α-hydroxyestrone (HE1), 17α-ethinylestradiol (EE2), mestranol (ME), β-estradiol 17-acetate (E2Ac), β-estradiol 3-sulfate (E2S3) and estrone 3-sulfate (E1S3), roxithromycin (ROX), clarithromycin (CLA) and erythromycin (ERY) as its antibiotic active form, in influents and effluents of sewage treatment plants (STP) by HPLC-MS/MS using the same sample and a single extraction and clean-up procedure. Such a multiresidue method is not described in the literature. Compliance within EU decision 657/2002/EC was desired for this project [87]. This recommendation requests analysing two MRM transitions per analyte to exclude false positive results.

4.1.2 Experimental to the method validation of hormones and antibiotics in wastewater

4.1.2.1 Materials

Water (HPLC grade) was obtained from Mallinckrodt Baker (Griesheim, Germany). Methanol, *tert*-butylmethylether (Suprasolv grade), ammonium acetate, acetone, ammonium hydroxide (analytical grade) and acetonitrile (Lichrosolv) were obtained from Merck (Darmstadt, Germany). Tetrahydofurane (analytical grade) was obtained from KMF-Laborchemie (Lohmar, Germany).

Estrone, estrone 2,4,16,16 - d_4, estriol, 16α-hydroxyestrone, β-estradiol 3-sulfate sodium salt, estone 3-sulfate potassium salt, erythromycin and roxithromycin were provided by Sigma-

Aldrich (Seelze, Germany). 17β-Estradiol hemihydrate, β-estradiol-17-acetate, 17α-ethinylestradiol, mestranol (Vetranal^{TM}) were obtained from Riedel-de Haën (Seelze, Germany). Estrone 3-sulfate 2,4,16,16 - d_4 sodium salt and 17α-ethinylestradiol 2,4,16,16 - d_4 were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Clarithromycin was provided by Promochem (Wesel, Germany). The synthesis of (E)-9-[O-(2-methyloxime)]-erythromycin is described by Schlüsener et al. 2003 [58] and in chapter 2.2.1.2.

4.1.2.2 Internal Standards

10 mg of 17α–ethinylestradiol - d_4 (EE2 - d_4) was dissolved in 90 mL acetone, the volume was calibrated to 100 mL with HPLC-water. 10 mg of estrone 3-sulfate 2,4,16,16 - d_4 sodium salt (E1S3 - d_4) was dissolved in 90 mL water and the volume was calibrated to 100 mL with acetone. The internal standard solution for the steroid hormones (IS-H) was made by dilution with methanol of these two stock solutions to a final concentration of 10 ng/µL. The internal standard for the analysis of the macrolide antibiotics was made by dissolving 10 mg (E)-9-[-O-(2-methyloxime)]- erythromycin in 100 mL acetonitrile (IS-A).

4.1.2.3 Solid Phase Extraction

The DVB-phobic Speedisk cartridges from Baker (8086, Bristol/PA, USA) were conditioned with 15 mL methanol followed by 15 mL HPLC-water. A solid-phase extraction manifold (IST, Grenzach-Wyhlen, Germany), with PTFE stopcock and outlet, was used. The wastewater samples (~1000 mL) were passed through the cartridge at a speed of 100 mL/min (vacuum) by means of a Speedisk sample remote adapter (Baker, Bristol/PA, USA). The exact volume of the water sample was determined by difference weighting. The cartridge was washed with 15 mL HPLC-water to remove ionic compounds and dried for 5 min by gently sucking air through the cartridge. The analytes were eluted from the cartridge with 15 mL *tert*-butylmethylether followed by 15 mL methanol in a 30 mL amber flask. 10 µL of internal standard solution for the analysis of steroid hormones (IS-H) and 10 µL internal standard solution for the analysis of macrolide antibiotics (IS-A) were added to the mixture. The flask was closed, shaken by hand for 10 seconds and stored at -18 °C prior to clean-up.

4.1.2.4 Size Exclusion Chromatography (SEC) clean-up

Matrix components with high molecular masses were removed by SEC. The wastewater extract was condensed to 1 mL at 60 °C and 35 mbar by a Büchi Syncore® Analyst 12 port evaporation unit (Essen, Germany). The residue was dissolved in 10 mL tetrahydrofurane (THF) and the volume was reduced again to 1 mL at 60 °C and 35 mbar and one millilitre of THF:acetone, (70:30, v/v) was added. The complete sample was injected onto the SEC column. The SEC system consisted of a G1379A vacuum degasser, a G1311A quaternary pump equipped with a relay bus card (Agilent, Waldbronn, Germany), a rheodyne 7725i manual injection valve with a 2 mL sample loop (Rheodyne, Bensheim, Germany) and a C2-2006D automatic valco valve (VICI AG, Schenkon, Switzerland) for fractionation. The size exclusion was performed on a Phenogel SEC column (21.2 mm i.d., length 300 mm, particle size 5 µm, 100 Å) (Phenomenex, Torrance, CA, USA) at ambient temperature in an air conditioned room at 24 °C.

Time synchronisation between the injection and the fraction valve was triggered using a short contact closure signal of the injection valve to the pump. Also the pump gave a short closure signal at time step 21 min and 45 min to the fractionation valve.

A mixture of THF:acetone, (70:30, v/v) was used as eluent at a flow rate of 3 mL/min. The first fraction from 0 min to 21 min, which contained the higher molecular weight compounds, was diverted to waste. The second fraction (21-45 min), which contained the hormones and antibiotics, was collected in a 100 mL amber flask. Afterwards, the column was rinsed for five minutes with the solvent prior to the next injection. The volume of the collected fraction was reduced to 1 mL by the Büchi evaporation unit at 60 °C and 35 mbar. The residue was dissolved in 10 mL methanol and the volume was reduced again to 1 mL at 60 °C and 35 mbar.

The best hard-cut and separation conditions of the SEC were studied with MS/MS detection while the SEC-column was connected via a micro-splitter valve (P-460S, Upchurch Scientific Inc., WA, USA) to the mass spectrometer (MS) and a split flow of 1 mL/min was introduced in the MS.

4.1.2.5 HPLC

The HPLC system consisted of a G1313A autosampler, a G1312A binary HPLC pump, a G1322A degasser and a G1316A column oven (all Agilent, Waldbronn, Germany).

Steroid hormones and conjugates

Separations were performed using a Synergi RP-MAX column (2 mm i.d., length 150 mm, particle size 4 µm) and a SecurityGuard (Phenomenex, Torrance, CA, USA) at 25 ± 1 °C. The flow rate was 0.2 mL/min. The HPLC gradient was established by mixing two mobile phases: phase A: pure water and phase B: pure methanol. Chromatographic separation was achieved with the following gradient: 0-1 min: 0% B; 1 min to 3 min: 0% to 70% B; 3 min to 23 min: 70% to 100% B; 23-29 min: 100% B; 29 min to 30 min: 100% to 0% B; 30-35 min: 0% B. Ten µL of each sample was injected.

Macrolide antibiotics

The HPLC separations of the macrolide antibiotics were performed using a Phenosphere-Next RP18 column (2 mm i.d., length 150 mm, particle size 3 µm) and a SecurityGuard at 25 ± 1 °C. The flow rate was 0.2 mL/min. The HPLC gradient was established by mixing two mobile phases: phase A, 10 mM aqueous ammonia acetate solution and phase B, pure acetonitrile. Chromatographic separation was achieved with the following gradient: 0-1 min 10% B, 1 min to 14 min 10% to 100% B, 14-29 min 100% B, 29 min to 30 min 100% to 10% B, 30-35 min 10% B. Ten µL of each sample were injected. An additional flow of 400 µL/min methanol was added after the separation by means of a second G1312A binary HPLC pump to improve the ionisation of the macrolide antibiotics in APCI mode.

4.1.2.6 Mass spectrometry

The triple quadrupole mass spectrometer (API 2000, Applied Biosystems, Darmstadt, Germany) was equipped with a TurboIonSpray source (ESI) and a Heated Nebuliser source (APCI).

Steroid hormones and conjugates (ESI)

The ESI source was operated under the following conditions: curtain gas (CUR), 40 psi; collision gas (CAD), 3 mTorr; ion spray voltage (IC), -4500 V; temperature (TEM), 400 °C; ion source gas 1 (GS1), 35 psi; ion source gas 2 (GS2), 70 psi; interface heater (ihe), on; focusing potential (FP), -350 V; entrance potential (EP), -10 V. The arrangement of the ESI-spray to the orifice was va, 5 mm and h, 3 mm (Table 27).

Steroid hormones and conjugates (APCI)

The APCI source was operated under the following conditions: curtain gas (CUR), 35 psi; collision gas (CAD), 3 mTorr; nebuliser current (NC), 2 µA; temperature (TEM), 450 °C; ion

source gas 1 (GS1), 60 psi; ion source gas 2 (GS2), 15 psi; interface heater (ihe), on; focusing potential (FP), 350 V; declustering potential (DP), 11 V; entrance potential (EP), 10 V.
The arrangement of the spray was vertical (va), 5 mm; horizontal (h), 3 mm to the orifice and the position of corona needle was va, 4 mm and h, 6 mm (Table 28).
Both soft ionisation modes were used in comparison.

Table 27: MS-conditions as well as retention time for the analysis of steroid hormones with electrospray ionisation in two time controlled experiments: (1) t_1 = 0-13.5 min, (2) t_2 = 13.5-35 min. Maximum variation in retention time was not larger than ± 0.1 min. HPLC conditions: Methanol: water, Synergi-RP Max

	RT [min]	precursor ion [amu]	product ion [amu]	dwell time [msec]	collision energy [eV]	declustering potential [V]	
E1S3	11.6	349	269	100	-44	-68	v
		349	143	100	-100	-68	q
E1S3-d_4	11.6	353	273	100	-45	-68	q
		353	147	100	-76	-68	v
HE1	12.3	285	145	100	-50	-68	v
		285	159	100	-50	-68	q
E3	12.5	287	171	100	-49	-68	v
		287	145	100	-58	-68	q
EE2	14.6	295	145	100	-60	-80	v
		295	159	100	-47	-80	v
EE2-d_4	14.6	299	174	100	-60	-80	v
		299	161	100	-47	-80	v
E1	14.9	269	145	100	-47	-140	v
		269	159	100	-47	-140	v
E1-d_4	14.9	273	147	100	-51	-140	
		273	161	100	-51	-140	
E2	14.8	271	145	100	-54	-140	v
		271	239	100	-53	-140	v
E2Ac	18.4	313	253	100	-38	-68	v
		313	145	100	-59		v

q: MRM transition used for quantification; v: MRM transition used for verification; amu: atomic mass unit

Table 28: MS-conditions as well as retention time for the analysis of steroid hormones with atmospheric pressure chemical ionisation. Maximum variation in retention time was not larger than ± 0.1 min. HPLC conditions: Methanol: water, Synergi-RP Max

	RT [min]	precursor ion [amu]	product ion [amu]	dwell time [msec]	collision energy [eV]	
HE1	12.4	287	251	100	21	v
		287	199	100	24	q
EE2	14.6	279	133	100	22	v
		279	159	100	27	q
EE2-d_4	14.6	283	135	100	28	q
		283	161	100	31	v
E1	14.9	271	159	100	29	q
		271	133	100	27	v
E1-d_4	14.9	275	161	100	29	
		275	135	100	32	
E2	14.8	255	159	100	24	v
		255	133	100	24	q
E2Ac	18.4	255	159	100	24	q
		255	133	100	24	v
ME	20.1	293	173	100	31	q
		293	147	100	27	v

q: MRM transition used for quantification; v: MRM transition used for verification; amu: atomic mass unit

Macrolide antibiotics (APCI)

The APCI source operated under the following conditions: curtain gas (CUR), 50 psi; collision gas (CAD), 3 mTorr; nebuliser current (NC), 5 µA; temperature (TEM), 500 °C; ion source gas 1 (GS1), 80 psi; ion source gas 2 (GS2), 35 psi; interface heater (ihe), on; focusing potential (FP), 360 V; declustering potential (DP), 20 V; entrance potential (EP), 10 V.

The arrangement of the spray was vertical (va), 5 mm; horizontal (h), 3 mm to the orifice and the position of corona needle was va, 4 mm and h, 6 mm.

Table 29: MS-conditions as well as retention time for the analysis of macrolide antibiotics with atmospheric pressure chemical ionisation. Maximum variation in retention time was not larger than ± 0.1 min. HPLC conditions: Acetonitrile: 10 mM aqueous ammonium acetate solution, Phenosphere-Next

	RT [min]	precursor ion [amu]	product ion [amu]	dwell time [msec]	collision energy [eV]	
Clarithromycin	13.7	748	158	120	45	q
		748	590	80	25	v
Erythromycin	12.8	734	158	120	45	q
		734	576	80	25	v
Roxithromycin	14.2	837	158	120	46	q
		837	679	80	35	v
Internal Standard (IS-A)	13.9	763	158	120	45	q
		763	605	80	25	v

q: MRM transition used for quantification; v: MRM transition used for verification; amu: atomic mass unit

MS calibration was performed up to m/z 1800 with mass resolution of quadrupole 1 and quadrupole 3, both were set to 0.7 Daltons. The data obtained were processed using Analyst^{TM} 1.4 software. To gain higher selectivity, multiple reaction monitoring (MRM) was chosen. MS/MS parameters were optimised in continuous flow mode, injecting 1,000 ng/mL standard solutions dissolved in methanol at a flow rate of 10 µL/min. The optimal collision energy (CE) and optimal declustering potential (DP) was determined by means of a software procedure controlling the automatic switching between the different voltages with a step size of 1 V/scan and a range from -5 to -130 V in positive mode and 5 to 130 V in negative mode for the CE and 0 to 200 V in positive mode and 0 to -200 V in negative mode for the DP. The MRM transitions as well as the individual declustering potential and collision energy voltage used for the analysis of steroids hormones in ESI and APCI mode are displayed in Table 27 and Table 28. The ‘q’ indicates the MRM transition that was used for quantification, all other transitions were used for verification (v).

The macrolide antibiotics are analysed more reliably in APCI as demonstrated for manure [58]. The parameters used in this study are demonstrated in Table 29.

4.1.2.7 Calibration

The calibration was performed as an internal standard calibration. A stock solution for the hormones was produced by dissolving 10 mg of the hormones in 30 mL acetone, 30 mL water and filled up to 100 mL with acetonitrile. A stock solution for the macrolide antibiotics was produced by dissolving 10 mg of the respective antibiotics in 100 mL acetonitrile. These stock solutions were stored at 4 °C in the dark and were renewed after 3 months. Calibration standards (1, 5, 10, 50, 100, 500, and 1,000 ng/mL) were made by serial dilution of the stock solution in methanol. Ten micro litre of each internal standard solution were added to each millilitre of the calibration standards. The calibration curves were calculated using a weighted (1/X) linear regression model.

4.1.3 Results and discussion to the method validation of hormones and antibiotics in wastewater

All analytes were separated by HPLC. The calibration graphs are linear in the range from the limit of quantification (LOQ) up to 1,000 ng/mL with correlation coefficients (R^2) better than 0.99.

Optimisation of ESI-signals:

The addition of buffers (ammonium acetate, ammonium formiate or ammonium hydroxide at varying concentrations, pH 3-11) to the mobile phase caused a decrease in the responses of the analytes due to lower ionisation ratios for the separation of steroid hormones. The use of pure methanol instead of pure acetonitrile as phase B gave factor 2-3 higher ionisation ratios in electrospray ionisation mode for these analytes. These results correspond with the literature [84, 88]. The postcolumn addition of a 40 mmol/L methanolic ammonia solution gave a decreasing of the response of the analytes in ESI negative mode. These results are different to Bartonti *et al*. [89] and Gentili *et al*. [90] who also used an API 2000 mass spectrometer and detected better ionisation ratios with the postcolumn addition of ammonia.

Optimisation of APCI signals:

The addition of ammonium acetate to the mobile phase of the macrolide antibiotics increased the ionisation performance of these analytes. Also the postcolumn addition of 400 µL/min methanol improved the ionisation of the macrolide antibiotics.

Thus, three HPLC-MS/MS methods are necessary for an optimal ionisation of all analytes.

4.1.3.1 The choice of internal standards

The use of estrone 2,4,16,16 - d_4 from Sigma-Aldrich as internal standard in an amount of 25 ng resulted in interferences with the corresponding undeuterated target compound up to concentrations of 3 ng/L, though Sigma-Aldrich claims an isotopic purity of 95 %. For this reason, the use of estrone 2,4,16,16 - d_4 was avoided in the final method.

The use of sodium estrone 3-sulfate 2,4,16,16 - d_4 and 17α-ethinylestradiol 2,4,16,16 - d_4 from Dr. Ehrenstorfer GmbH as internal standards gave no interference with the corresponding undeuterated target compound. E1S3, E2S3 and E3 were quantified with sodium estrone 3-sulfate 2,4,16,16 - d_4 as internal standard. 17α-ethinylestradiol 2,4,16,16 - d_4 was used to quantify E1, E2, EE2, ME, HE1 and E2Ac.

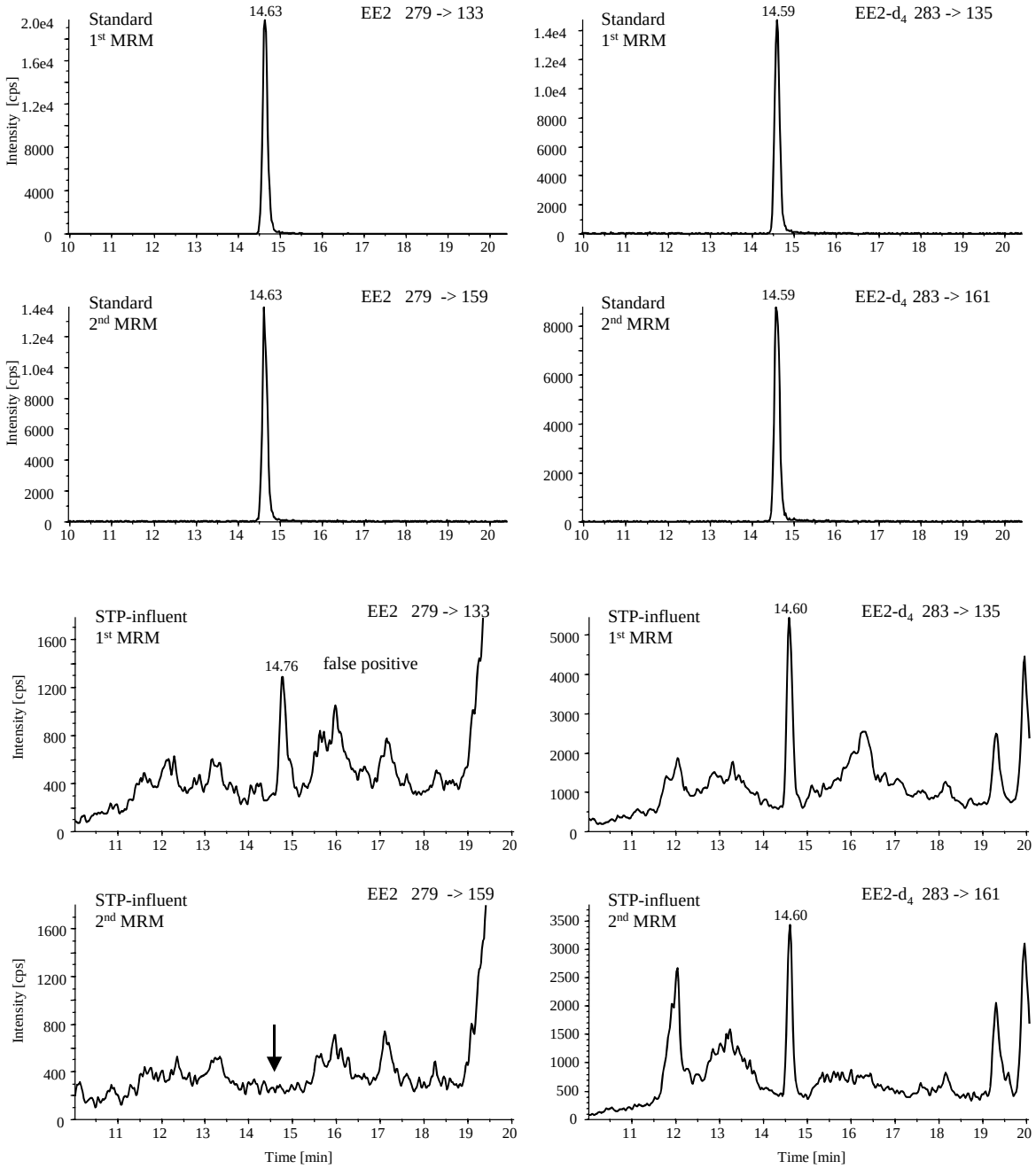
The use of (E)-9-[-O-(2-methyloxime)]- erythromycin as internal standard for the quantification of the macrolide antibiotics gave no blank problems.

4.1.3.2 Peak identification

EU decision 2002/657/L221 requires four identification points for the identification in HPLC-MS/MS analysis [87]. By using the HPLC-MS/MS technique, each precursor ion results in one identification point, while each transition product ion is counted as 1.5 points. Therefore, four points can be obtained by measuring one precursor and two product ions. Additionally the ratio of the chromatographic retention time of the analyte to that of the internal standard, the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ± 2.5 % [87].

Figure 24 shows the MRM chromatograms of ethinylestradiol and its internal standard, ethinylestradiol - d_4, in a standard solution and a STP-influent sample. By using only the first MRM transition and the ± 2.5 % tolerance of the retention time criterion to identify the respective peak resulted in a false positive identification. Only using a second MRM transition for verification gave a correct result.

Figure 24: MRM chromatograms of 17α-ethinylestradiol and its internal standard, ethinylestradiol - d_4, in an standard solution and a STP-influent sample. A false positive determination is detected by the missing peak in the second MRM.



4.1.3.3 Matrix effects

A main problem in the quantitative liquid chromatography coupled with tandem mass spectrometry is the unexpected matrix effect. Different strategies are established to compensate these matrix effects:

1) Matrix calibration:

This is an internal standard calibration in the presence of a uncontaminated matrix to account matrix effects [50, 51]. This matrix is produced from uncontaminated samples by the same sample preparation, which is used for the analysis of the samples and is added to the calibration standards. A matrix calibration is working well, if there is access to uncontaminated sample matrix. It is difficult to obtain matrix wastewater samples free of natural steroid hormones and antibiotics.

2) Standard addition:

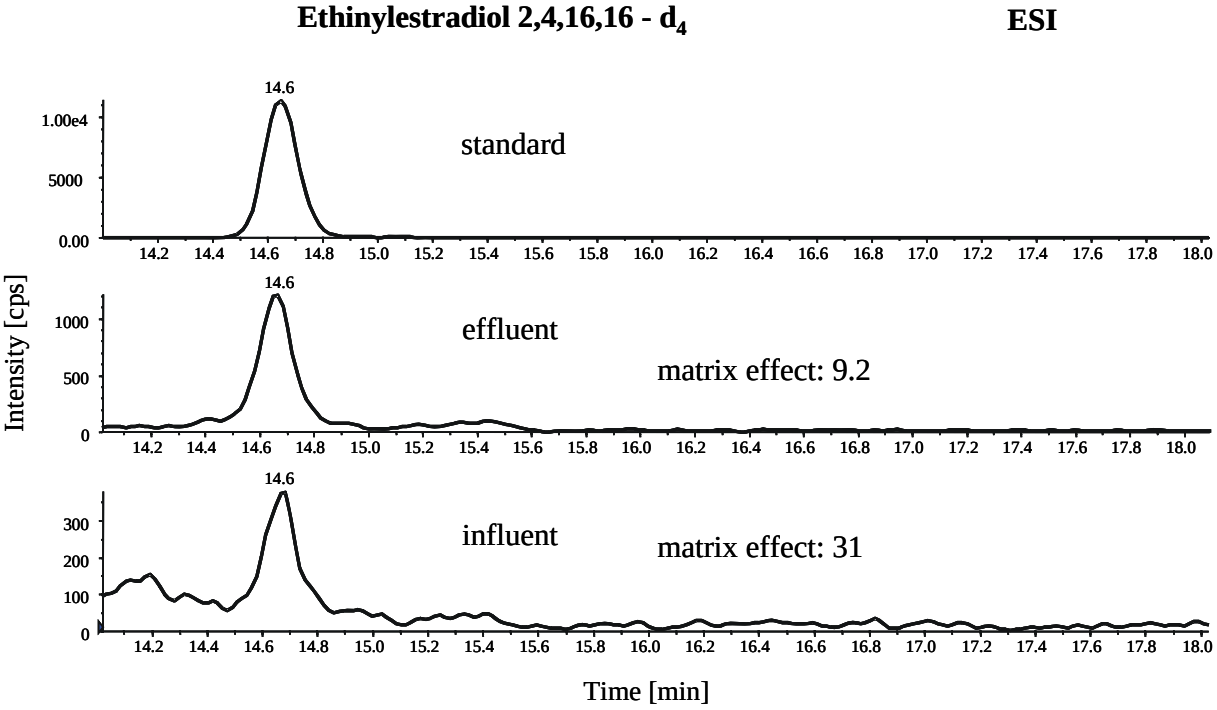
The sample is divided into several sub-samples and standard calibration solution is added to the sub-samples [91, 92]. As a result, a calibration curve is generated for each sample. This method is working well with a low number of samples but it is impracticable with high sample throughput, as it causes multiplication of analysis time because the number of samples is multiplied by the number of standards.

3) Isotope dilution:

The quantification with isotopic labelled internal standards. These standards have the same chemical nature, co-elute with the respective analyte and the matrix. Thus the same effect occurs to the internal standard as well as to the analyte. A disadvantage is the less availability of these standards.

All three options can compensate the matrix effects, but none of these options reduce these effects. Figure 25 shows the matrix effects of 17α-ethinylestradiol 2,4,16,16 - d_4 at a concentration of 100 ng/mL with electrospray ionisation (ESI). In spite of an elaborated clean-up procedure this internal standard shows a 31 times lower signal in an influent sample due to matrix effects while a 9-fold decrease is observed for effluent samples. As a consequence of these high matrix effects quantification is difficult at concentrations levels near the original LOQ. This implies that the limits of quantification are raising.

Figure 25: ESI MRM chromatograms of 17α-ethinylestradiol 2,4,16,16 - d_4 in a standard solution in comparison to extracts of effluent and influent from STP samples (peak height) spiked to 100 ng/mL, each. The resulting matrix effect is calculated by dividing the peak height (intensity) for the IS of the standard solution by the peak height for the IS of the respective sample extract.

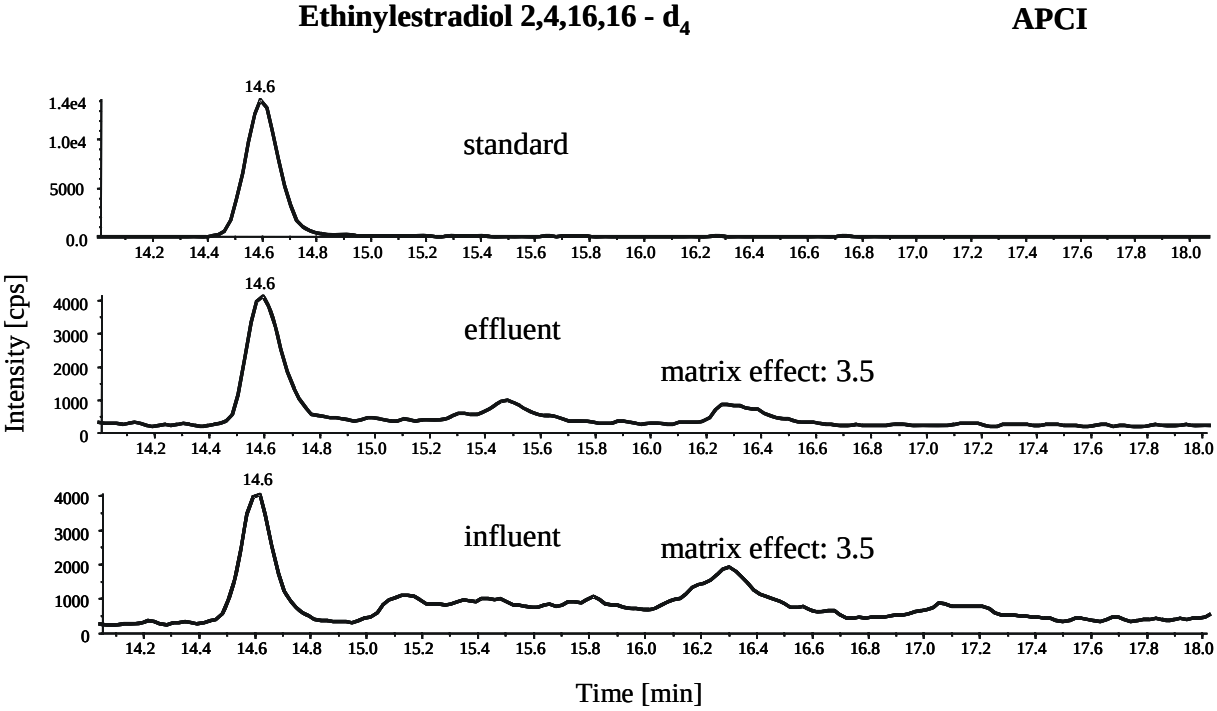


To reduce these matrix effects chemical ionisation at atmospheric pressure (APCI) can be utilised successfully. Figure 26 shows the chromatograms of 17α-ethinylestradiol 2,4,16,16 - d_4 in the same samples (standard, influent and effluent) as discussed above for ESI. Compared to the standard solution the ionisation of EE2-d_4 in the influent and effluent was only a factor 3.5 smaller while in ESI this effect was factor 31. Thus matrix effects in APCI-mode were about factor 3-10 less pronounced than in the ESI-mode. Similar effects have been observed for different matrices and ion sources from other manufactures in the literature [58, 91].

Thus the atmospheric pressure chemical ionisation is preferable to the electrospray ionisation for the analysis of steroid hormones in wastewater samples. Unfortunately, not all analytes could be ionised by APCI, therefore E1S3, E2S3 and E3 were quantified by ESI-MS/MS. However, the matrix effects for these three hormones were much lower than for the later eluting steroid hormones.

The comparison of ESI and APCI for the analysis of macrolide antibiotics is described in Schlüsener *et al.* [58]. It revealed similar results as obtained in this study focussing on hormones.

Figure 26: APCI MRM chromatograms of 17α-ethinylestradiol 2,4,16,16 - d_4 in a standard solution in comparison to extracts of effluent and influent from STP samples (peak height) spiked to 100 ng/mL, each. The resulting matrix effect is calculated by dividing the peak height (intensity) for the IS of the standard solution by the peak height for the IS of the respective sample extract.



4.1.3.4 Validation of the method

The method was primarily validated by spiking 1 L of tap water with the stock solutions to produce concentrations of 1, 3, 10, 30, 100, 300 and 1,000 ng/L. The following sample preparation, extraction and clean-up was identical to the procedures described above. These recovery experiments for hormones and antibiotics were carried out at seven concentration levels in triplicate.

The recovery rates are given in Table 30. Since there was no significant concentration (1, 3, 10, 30, 100, 300 and 1,000 ng/L) dependency of the recovery rate, the values of all experiments were averaged. The limit of quantification (LOQ) was defined as a signal-to-noise ratio of 10:1 and the limit of detection (LOD) as a signal-to-noise ratio of 3:1. The signal-to-noise ratios of the LOD and LOQ were taken from the chromatograms of the recovery rates.

Mean recovery rates of the steroid hormones in APCI-mode ranged from 82% (RSD 12%) to 109% (RSD 15%). Mean recoveries of 58% (RSD 24%) for estriol, 95% (RSD 13%) and 92%

(RSD 9%) for the hormone-sulfates were obtained using the electrospray ionisation. This method was also applied to β-estradiol 17-β-D-glucuronide but it did not give constant recovery rates for this compound. The mean recovery rates for the macrolide antibiotics were 79 % (RSD 11%) for roxithromycin, 82 % (RSD 7%) for clarithromycin and 100 % (RSD 15%) for erythromycin (Table 30).

4.1.3.5 Stability of the method in respect of matrix and during sample transport and storage

To proof the stability of the analytical method eleven 1 L STP influent samples were spiked with 100 ng of the respective analytes. Five spiked samples were extracted immediately, five samples were stored in the dark at 4 °C for 48 hours before the extraction. The sample preparation, extraction and clean-up of these ten samples were identical to the procedures described above. The last sample was extracted directly after spiking, but this sample received no SEC clean-up. Another two unspiked influent samples were also analysed to determine the blank levels. Table 30 shows the recovery rates of the spiked influent samples. The recovery rates of the spiked wastewater samples were all identical to those obtained from spiked tap water within the precision of the method.

In comparison with stored samples only a degradation of mestranol and β-estradiol 17-acetate was observable in the same experiment. The “recovery” was reduced from 97% to 68% for mestranol while those of β-estradiol-17-acetate changed from 112% to 58%. β-Estradiol 3-sulfate shows a maximal “increase” of 10 % of the concentration. The recovery rates of all other analytes were identical to those extracted immediately from wastewater and tap water.

4.1.3.6 Comparison between samples with and without clean-up

The different recovery rates of the uncleaned sample (without SEC) in comparison to the cleaned samples based on the different matrix effects of the internal standards to their respective analytes. In comparison to samples that were processed with clean-up to those that were processed without clean-up revealed lower results considering: estrone, the macrolide antibiotics and estriol using APCI and ESI respectively.

The limit of quantification, defined as the signal to noise ratio of 10:1, in wastewater influent samples were about maximal two times higher than in tap water samples in APCI mode. In ESI mode the LOQ increased by the factor of 7 (Table 30).

Table 30: Mean recovery rate as well as standard deviation, relative standard deviation (RSD), and limit of quantification (LOQ), (three extractions, repetitions for each concentration level) of hormones and antibiotics in tap water. Recoveries were determined at concentrations of 1, 3, 10, 30, 100, 300 and 1000 ng/L water. Also mean recovery rate ± standard deviation of five spiked STP influent samples, mean recovery ± standard deviation of five spiked STP influent samples stored for 48h at 4°C, recovery of one spiked influent sample without SEC clean-up and limit of quantification (LOQ) of hormones and antibiotics in wastewater influents. LOQ: S/N = 10:1

	Mean recovery [%] tap water n=21	**RSD [%]**	**Mean recovery [%] influent n=5**	**Mean recovery after 48 h [%] influent n=5**	**Recovery without SEC [%] n=1**	**LOQ [ng/L] effluent tap water**	**LOQ [ng/L] influent**
Hormones (APCI)							
Mestranol	105±12	11	97±12	68±7	73	3	6
16α-Hydroxyestrone	74±15	21	86±13	75±8	87	8	8
17β-Estradiol	98±9	9	83±8	88±7	87	8	8
Estrone	105±16	15	90±12	113±11	64	2	4
17α-Ethinylestradiol	83±6	7	85±9	82±4	82	6	6
17β-Estradiol-17-acetate	109±15	14	112±17	51±6	104	1.5	3
Hormones (ESI)							
β-Estradiol 3-sulfate	92±8	9	95±7	119±11	98	1.8	28
Estrone 3-sulfate	95±12	13	77±8	76±7	74	0.6	4
Estriol	58±14	24	63±19	43±9	16	15	35
Antibiotics (APCI)							
Clarithromycin	82±6	7	91±7	91±12	30	2	2
Erythromycin	100±15	15	82±9	81±9	13	6	6
Roxithromycin	79±8	11	92±9	86±23	33	6	6

n: number of spiked extracted samples

4.1.3.7 Application to environmental samples

The method was tested for several wastewater samples in order to investigate the fate of hormones and antibiotics during wastewater treatment. Wastewater from a sewage treatment plant in the Ruhr region of North Rhine Westphalia (Germany) with 250,000 inhabitant equivalent values was sampled during four days. The samples were taken automatically as 24-hour composite samples at the inflow and effluent of a sewage treatment plant. The samples were refrigerated at 4 °C during the 24 h intervals. They were transported to the laboratory immediately after sampling and extracted within 6 hours after arrival. The samples were generally extracted on the same day. When it was not possible to extract the hormones immediately, the samples were stored at 4 °C for two days maximum. All extractions were performed in duplicate.

The steroid hormone 17β-estradiol was found with concentration up to 22 ng/L in influents and 8.6 ng/L in effluents (Table 31). The metabolites of 17β-estradiol, estrone, 16α-hydroxyestrone and estriol were determined with maximal concentrations of 87 ng/L (E1), 90 ng/L (HE1) and 470 ng/L (E3) in influents and 5.3 ng/L (E1), 14 ng/L (HE1) and 99 ng/L (E3) in effluents. These results were in the same order of magnitude as the results of Bartoni *et al.* [89] and Gentili *et al.* [90] who measured steroid hormones in influents and effluents of different sewage treatment plants in Italy. The conjugates of the steroid hormones were found at maximal concentrations of 8 - 28 ng/L (E2S3) and 23 ng/L (E1S3) in influents in the Ruhr region. These results correspond with Gentili *et al.* [90] who found E1S3 in concentrations up to 3.9 ng/L. Estradiol 3-sulfate was found at maximal concentrations of 37 ng / L (E2S3) and estrone 3-sulfate 14 ng/L (E1S3) in effluents in the Ruhr region. These results match with the results of Isobe *et al*. [93] who analysed nine steroid hormone conjugates in effluents of Japanese STPs and found only E1S3 and E2S3 in concentrations of 0.3-2 ng/L. The contraceptives mestranol, 17α-ethinylestradiol and β-estradiol 17-acetate were neither detected in inflow nor in effluent samples of this sewage treatment plant in the Ruhr region. The concentrations of 17α-ethinylestradiol in wastewater were below the limit of detection (2 ng/L). Similar results considering inflow and outflow data for estrone and estriol were obtained by Bartoni *et al.* [89]. However, Bartoni *et al.* [89] found 17α-ethinylestradiol up to concentration of 0.4 - 13 ng/L in influents of Italian STPs.

Maximal concentrations of macrolide antibiotics in influents were found to be 370 ng/L (CLA), 160 ng/L (ROX) and 1,200 ng/L for erythromycin. In effluents concentrations of antibiotics were 230 ng/L (CLA), 130 ng/L (ROX) and 320 ng/L for erythromycin. Compared with the results of an other German group that measured only anhydroerythromycin [15] these

values are in same range. In comparison with values from STPs of Switzerland [35] the data from the Ruhr region were higher. Table 31 shows the concentrations in inflow and effluent samples of the hormones and antibiotics at four different sampling days with different weather conditions.

Table 31: Concentrations of influents and effluents at four different sampling days of a STP in the Ruhr region of North Rhine Westphalia (Germany) with 250,000 inhabitant equivalent values and different weather conditions. The deviation based on the relative standard deviation of the validated method.

		day 1 [ng/L]	**day 2 [ng/L]**	**day 3 [ng/L]**	**day 4 [ng/L]**
17β-Estradiol	influent	18±2	12±1	11±1	22±2
	effluent	-	-	8.6±0.8	2.4-8*
Estrone	influent	45±7	87±13	42±6	32±5
	effluent	4.6±0.7	5.3±0.8	-	2.0±0.3
16α-Hydroxyestrone	influent	13±2	90±14	18±3	9.5±1.4
	effluent	2.4-8*	6.7±1.0	14±2	6.8±1.0
Estriol	influent	54±13	470±110	66±16	55±13
	effluent	20±5	99±24	4.5-15*	-
β-Estradiol 3-sulfate	influent	-	8-28*	-	-
	effluent	-	6.4±0.6	3.0±0.3	37±3
Estrone 3-sulfate	influent	1.2-4*	23±3	12±2	5.3±0.7
	effluent	4.1±0.5	3.0±0.4	1.9±0.3	14±2
Clarithromycin	influent	180±12	370±26	210±15	81±6
	effluent	130±9	230±16	220±16	81±6
Erythromycin	influent	180±27	1200±180	850±130	97±15
	effluent	180±27	320±48	270±41	66±10
Roxithromycin	influent	96±13	160±23	110±15	54±8
	effluent	63±9	110±15	130±18	38±5

* : Value <LOQ ; - :<LOD; Ethinylestradiol < LOD (2 ng/L)

4.1.4 Conclusions to the method validation of hormones and antibiotics in wastewater

A reliable multiresidue method with low LOQs has been developed to analyse steroid hormones, their conjugates, the synthetic contraceptives and macrolide antibiotics unaltered in unfiltered influents and effluents of sewage treatment plants. This method can be used to investigate the fate of these compounds in various steps of wastewater treatment.

Solid phase extraction followed by a SEC clean-up step resulted in sufficiently clean extracts, which were analysed by HPLC-APCI-MS/MS and HPLC-ESI-MS/MS. The electrospray ionisation was compared to atmospheric pressure chemical ionisation considering matrix effects in HPLC-MS/MS analysis. The atmospheric pressure chemical ionisation mode should be preferred to electrospray ionisation even though less sensitivity is obtained in standard solutions. The reduction of matrix effects in APCI mode is an advantage of this ion source.

For the analysis of hormones in wastewater, it is important to follow on the EU decision 2002/657/L221 to prevent false positive results. The use of a second MRM is essential for verification.

4.2 Fate of antibiotics and steroid hormones during wastewater treatment

4.2.1 Introduction to the fate of hormones and antibiotics in wastewater

Wastewater is treated predominantly by a combination of mechanical, biological and chemical treatment steps in Germany before a discharge into surface waters occurs. The elimination of nutrients and harmful substances are thus performed in multistage processes of municipal sewage treatment plants. However, every STP is a unique system with different arrangements of mechanical, biological and chemical processes i.e. nitrification, denitrification, phosphate precipitation, neutralisation, activated-sludge and trickling filter techniques. Different executions and arrangements of these techniques result in different elimination rates of organic pollutions.

For the municipal sewage treatment plant operators as well as planners of such plants it is of outstanding relevance to know whether and how the elimination of organic pollutants in STPs is performed. It is an advantage to know by which procedural modification a better elimination of relevant harmful substances occurs.

A part of the published literature tries to describe the elimination of steroid hormones and estrogenic active compound in only one STP [94, 95] at two sampling days and two-times at one sampling day respectively. Ternes *et al.* tested two STPs in Germany and Brazil over a period of six days [80]. They found elimination rates of 83 % for estrone and 99.9 % for 17 β-estradiol in a German plant. Schullerer *et al.* examined three plants in Baden-Württemberg, Germany [96]. One plant was tested over a period of seven days. Elimination rates ranged from 87 %- 92 % for steroid hormones. The other two plants were only sampled on one day.

In this study, three STPs in North Rhine-Westphalia, Germany, with different concepts of wastewater treatment were chosen in order to investigate their efficiency in eliminating of macrolide antibiotics, steroid hormones, oral contraceptives and hormone conjugates over four weeks, considering hydraulic retention times as well as sludge retention times.

4.2.2 Experimental to the fate of hormones and antibiotics in wastewater

Chemicals, solid phase extraction, SEC clean-up, HPLC and mass spectrometry conditions were the same as described previously (chapter 4.1.2.1).

4.2.2.1 Description of the sample sites

STP 1

Sewage treatment plant 1 is a middle-sized plant with 250,000 inhabitant equivalent values. The average wastewater inflow per day is 70,000 m^3. This plant is equipped with an aerated grit chamber, a primary settling tank, two aeration basins, a circular aeration basin and a final settling tank. The denitrification step is disposed upstream while the precipitation of phosphate occurs by means of ferric salts. The inflow was sampled before the processed water of the sludge dewatering is added to the wastewater. The effluent was tested before the cleaned wastewater was discharged into the river. Figure 27 gives a more detailed insight into the wastewater treatment process of this STP. The samples were taken during the period from 31.08.04 till 26.09.04.

STP 2

Sewage treatment plant 2 is smaller than STP 1 with 64,000 inhabitant equivalent values. The average wastewater inflow per day is 12,000 m^3. This plant is equipped with a neutralization line, an aerated grit chamber, a preliminary settling tank, a trickling filter and a final settling tank and finally a postdenitrification step. The precipitation of phosphate occurs by means of ferric salts after the trickling filters. The inflow was sampled after the screen cleaner and before the neutralization line. The effluent was tested directly after the denitrification and before the tertiary ponds. After the tertiary ponds the wastewater was discharged into the river. The technical sketch of this STP gives a more detailed insight into this wastewater treatment process (Figure 28). The samples were taken during the period from 28.02.05 till 30.03.05.

Figure 27: Technical sketch of STP 1 with 250,000 inhabitant equivalent values.

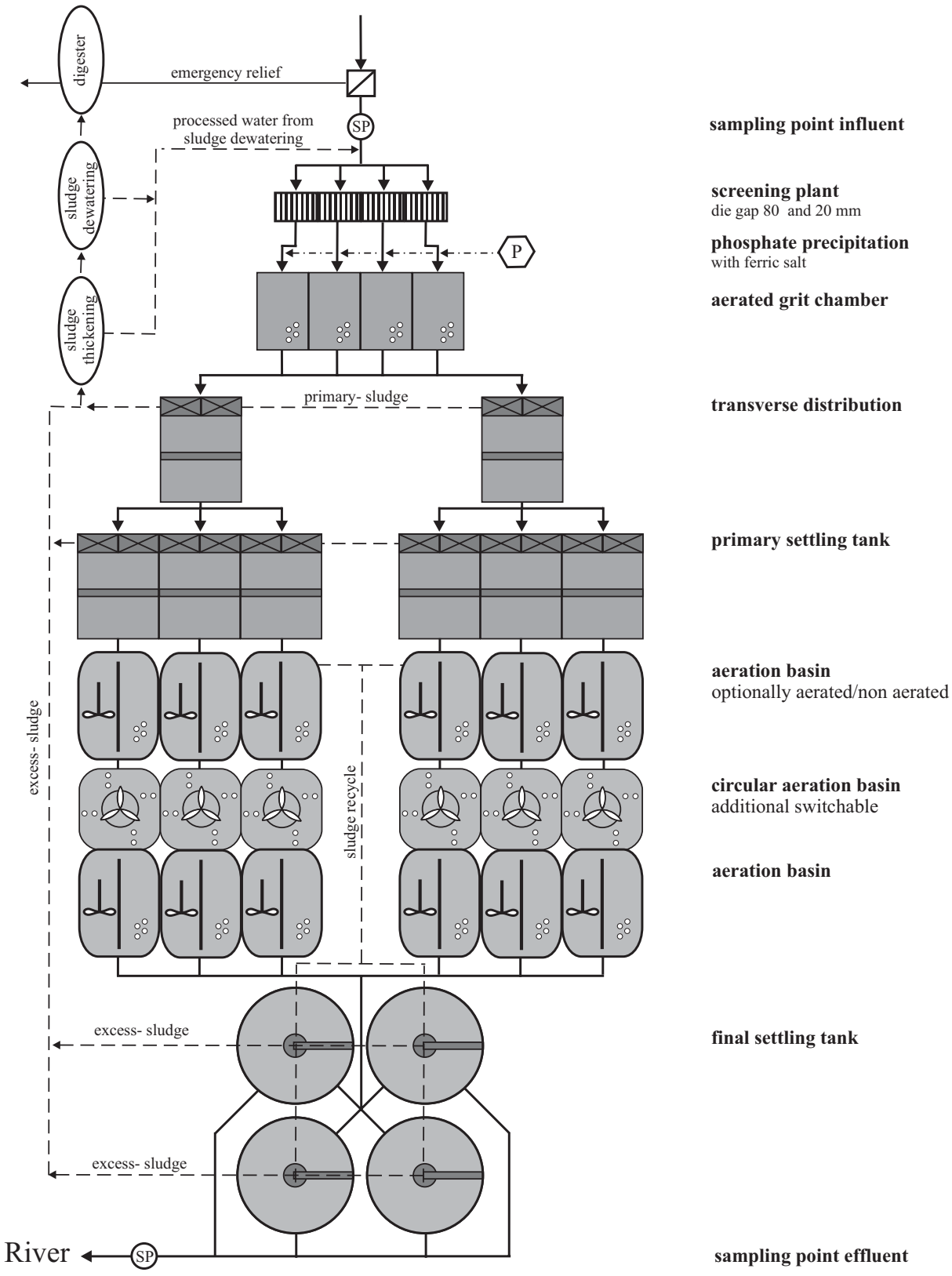
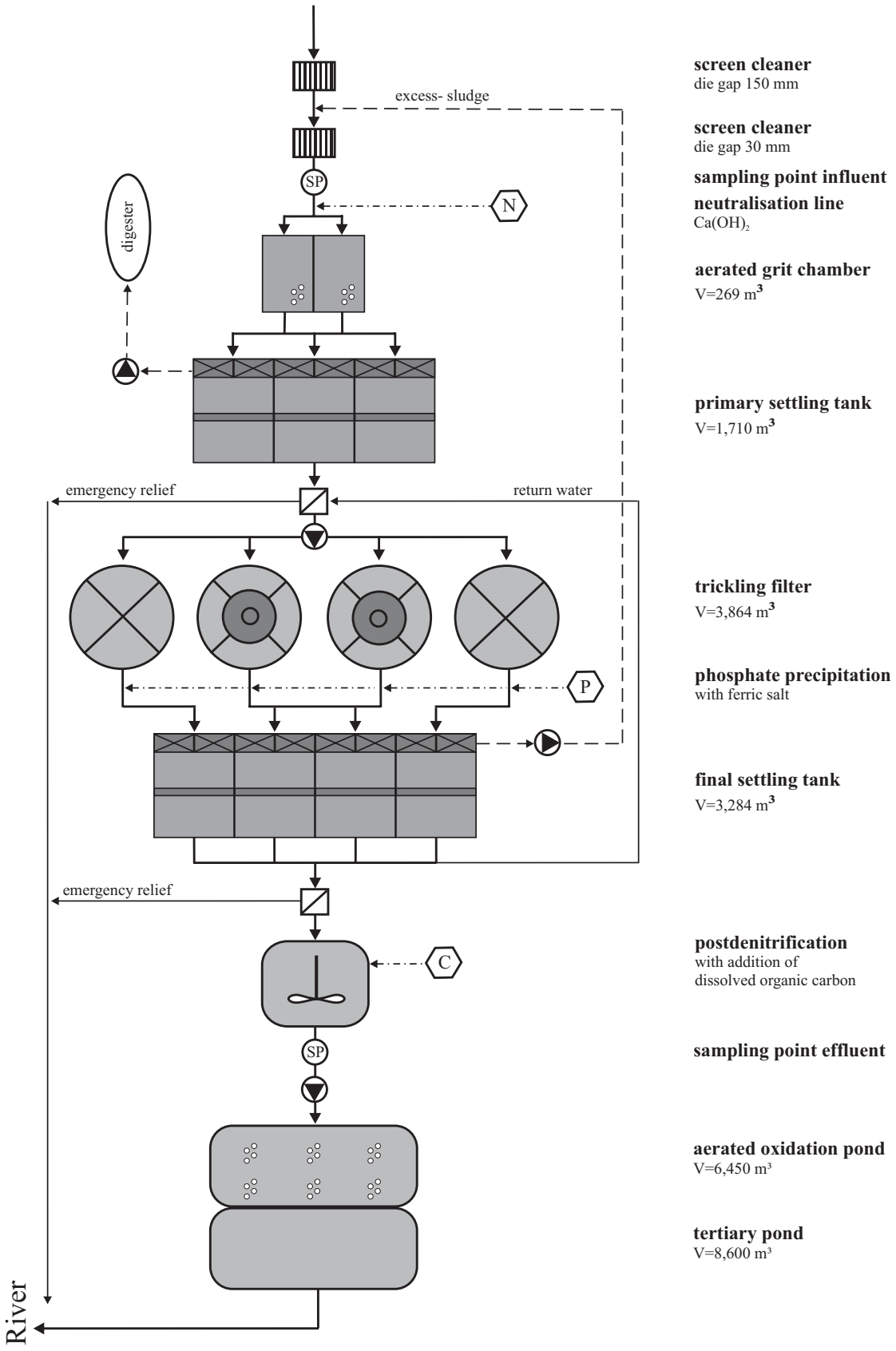


Figure 28: Technical sketch of STP 2 with 64,000 inhabitant equivalent values.

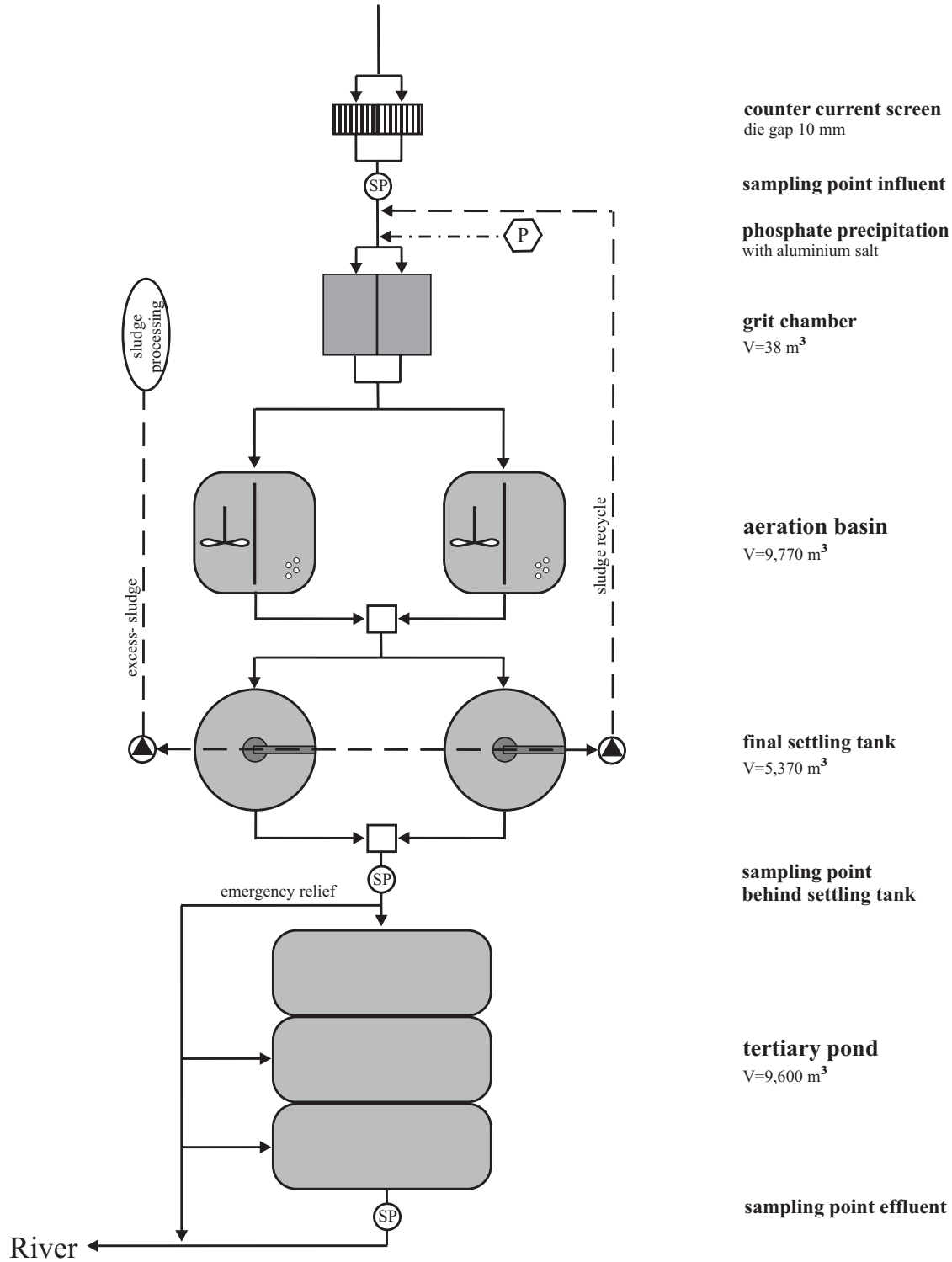


STP 3

Sewage treatment plant 3 is a small plant with 32,000 inhabitant equivalent values. The average wastewater inflow per day is 13,000 m^3. This plant is equipped with a grit chamber, a aeration basin with simultaneous nitrification and denitrification and a final settling tank. The precipitation of phosphate occurs by means of aluminium salts at the inflow. The inflow was sampled after the screen cleaner and before the phosphate precipitation. The effluent was tested before the cleaned wastewater was discharged into the river. Additionally, every three days the effluent of the final settling tanks was also tested. A detailed insight into this wastewater treatment process is shown in Figure 29. The samples were taken during the period from 06.06.05 till 03.07.05.
Supplementary, a 24-h characteristic curve were taken in 2-h steps at the inflow of this plant.

All samples were taken automatically as 24-hour composite samples. The samples were refrigerated at 4 °C during the 24 h intervals. They were transported to the laboratory immediately after sampling and extracted within 6 hours after arrival. The samples were generally extracted on the same day. When it was not possible to extract the hormones and antibiotics immediately, the samples were stored at 4 °C for two days maximum. All samples were extracted in duplicates.

Figure 29: Technical sketch of STP 3 with 32,000 inhabitant equivalent values.



4.2.3 Results and discussion to the fate of hormones and antibiotics in wastewater

During the complete sampling periods of all three STPs, no mestranol, 17α-ehinylestradiol and β-estradiol 17-acetate could be detected. Detailed information of the concentration and day by day load is presented in the supplement (chapter 10) and will be presented in the final report of the project BASPiK [97]

4.2.3.1 STP 1

Steroid Hormones

The daily inflow load of the steroid hormones ranged from 0.1 g up to 14 g during dry weather conditions, depending on the different types of hormones. During the rainfalls, the wastewater flow-rate roses up from 40,000 m³/d to 180,000 m³/d. Also the inflow load of the steroid hormones rose up to 20 g/d respectively. As the steroid hormones are excreted by humans via the urine and the excretion rate does not raise up during rainfalls, the higher loads in the wastewater flow of this hormones stems from hormones bound to sediments which were transported to the STP due to high flow-rates in the sewers.

Figure 30: Daily loads of estrone in the inflow and the effluent of STP 1 over the sampling period. Additionally the wastewater flow-rate during the sampling period is given.

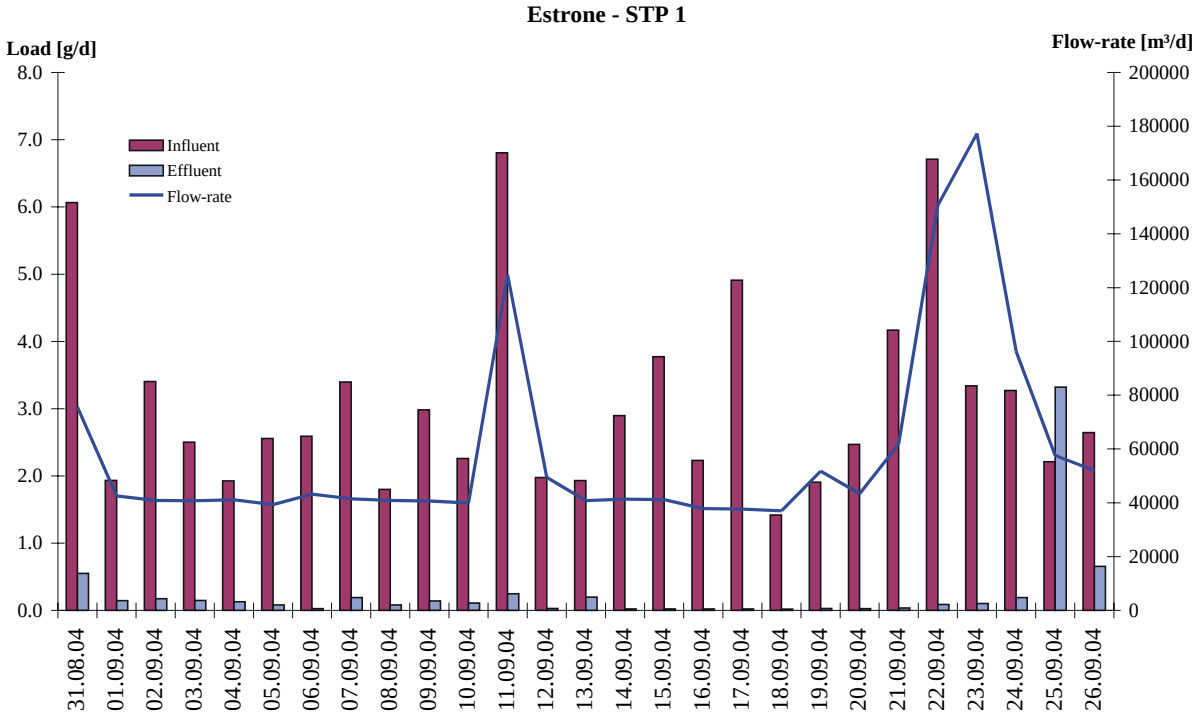
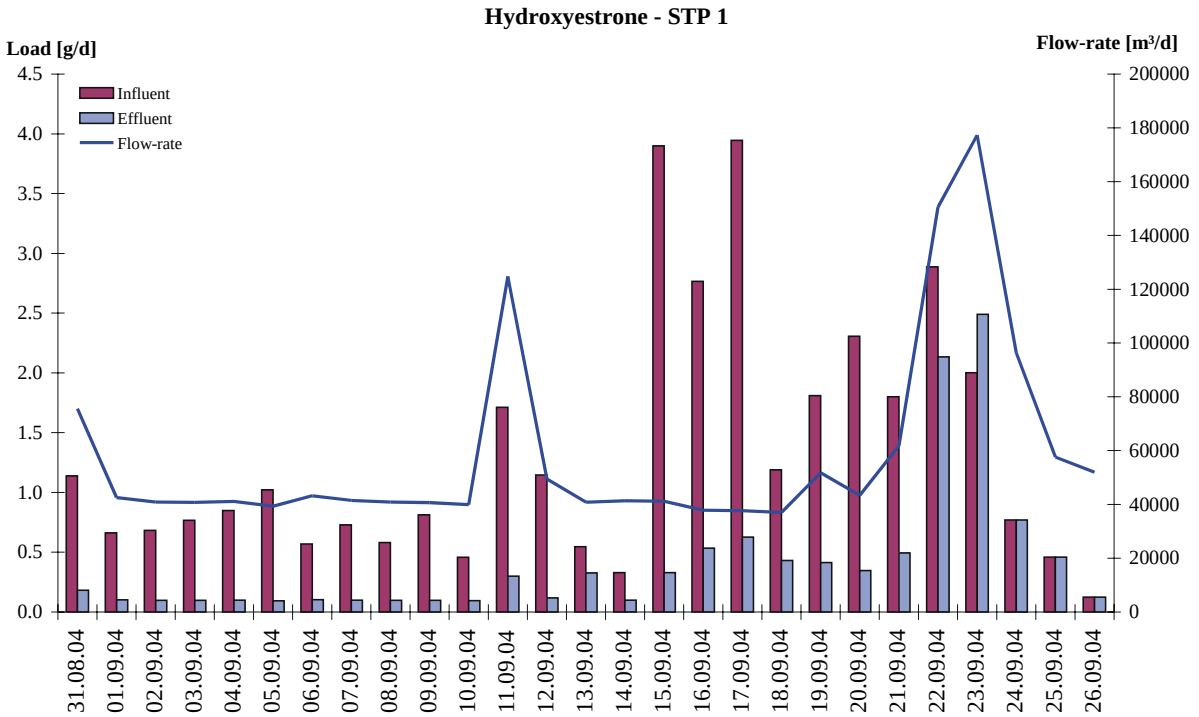


Figure 31: Daily loads of hydroxyestrone in the inflow and the effluent of STP 1 over the sampling period. Additionally the wastewater flow-rate during the sampling period is given.



As examples for all steroid hormones Figure 30 and Figure 31 show the daily loads of estrone and hydroxyestrone during the sampling period, respectively.

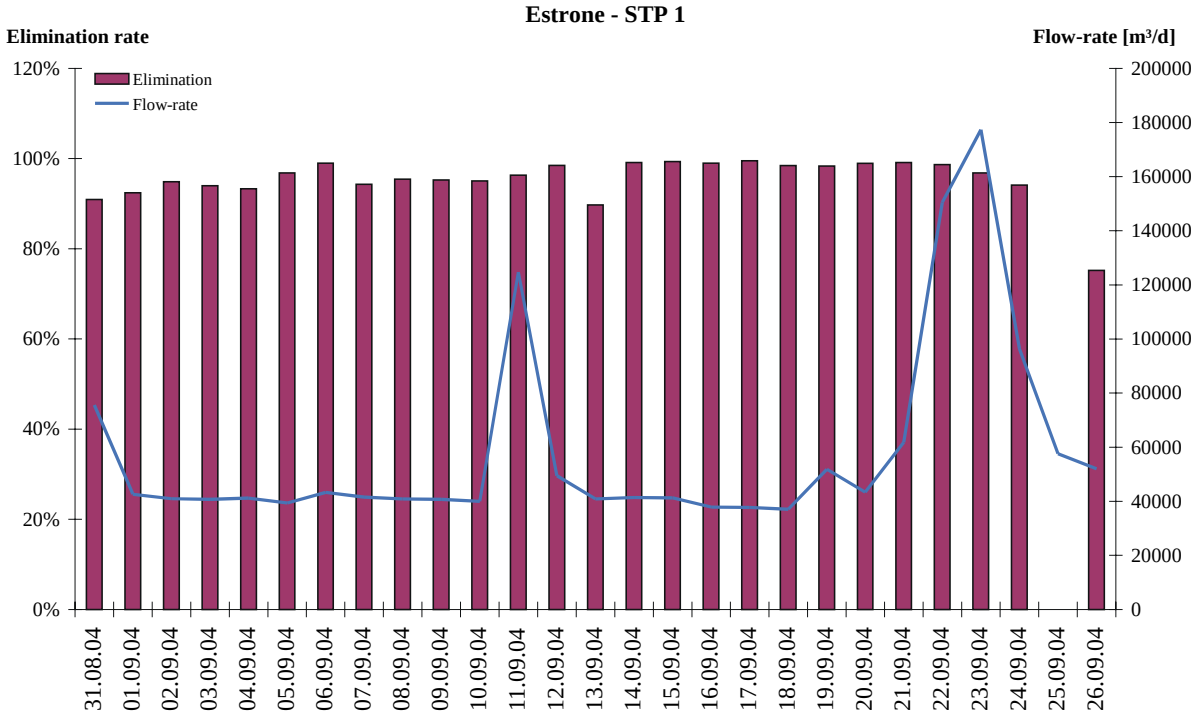
The concentrations ranged from 19 - 130 ng/L for estrone, <LOD – 110 ng/L for hydroxyestrone, up to 68 ng/L for estradiol, 510 ng/L maximum for estriol, up to 12 ng/L for estrone 3-sulfate and <LOD – 28 ng/L for β-estradiol 3-sulfate in influents. The effluents had generally lower concentrations.

By comparing the loads of the influents with the load of the effluents, an elimination rate was calculated (Table 32). While the elimination of estrone is nearly 100 % the elimination of the other hormones ranged from 41% - 75 %, excepting β-estradiol 3-sulfate. No significant elimination could be observed during the wastewater treatment in this plant.

Table 32: Elimination rates of steroid hormones and macrolide antibiotics in the three investigated STPs. The elimination rates based on the complete mass flow rates during the sampling period. The standard deviation based on the SD of the validated method and Gaussian error propagation. Negative values indicate "generation". Positive values indicate "elimination".

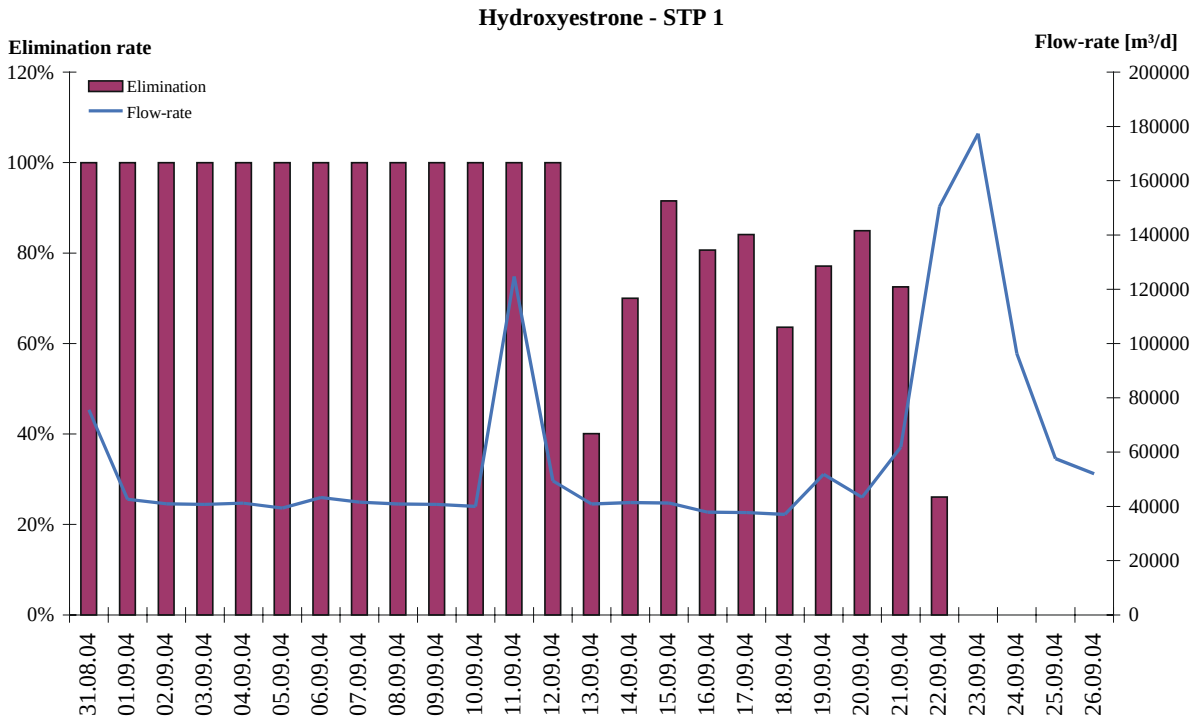
	STP 1 Elimination [%]	STP 2 Elimination [%]	STP 3 Elimination [%]
Estrone	92 ± 2	-72 ± 36	50 ± 11
16 α-Hydroxyestrone	69 ± 9	64 ± 11	82 ± 5
17β-Estradiol	75 ± 3	-53 ± 19	26 ± 9
Estriol	58 ± 14	34 ± 23	69 ± 11
Estrone 3-sulfate	13 ± 16	-360 ± 85	73 ± 5
β-Estradiol 3-sulfate	41 ± 14	-74 ± 15	13 ± 10
Erythromycin	23 ± 16	0 ± 21	15 ± 18
Clarithromycin	-14 ± 10	-3 ± 9	-7 ± 9
Roxithromycin	-19 ± 18	-22 ± 19	-22 ± 27

Figure 32: Elimination rates of estrone in STP 1 during the sampling period in comparison to the wastewater flow-rate.



The day to day variation of the elimination rate of estrone in this STP is nearly uninfluenced by rainfalls (Figure 32), except the heavy rainfalls at the end of the sampling period can disturb the elimination of estrone in this STP. Hydroxyestrone as an example for all other steroid hormones shows a decrease of the elimination efficiency during rainfalls (Figure 33). In the beginning of the sampling period the elimination rate of hydroxyestrone is nearly 100 %. After the rain event at 11.09.04 the elimination rates ranges from 60% - 90%, while the elimination was complete disabled during and after the rain event from 21.09-26.09.04.
The biological step of the STP was not able to handle the large inflow of wastewater considering the elimination of this compound. Maybe the bacteria which were responsible for the elimination of hormones needs time to adapted on the new situation after rainfalls.

Figure 33: Elimination rates of hydroxyestrone in STP 1 during the sampling period in comparison to the wastewater flow-rate.

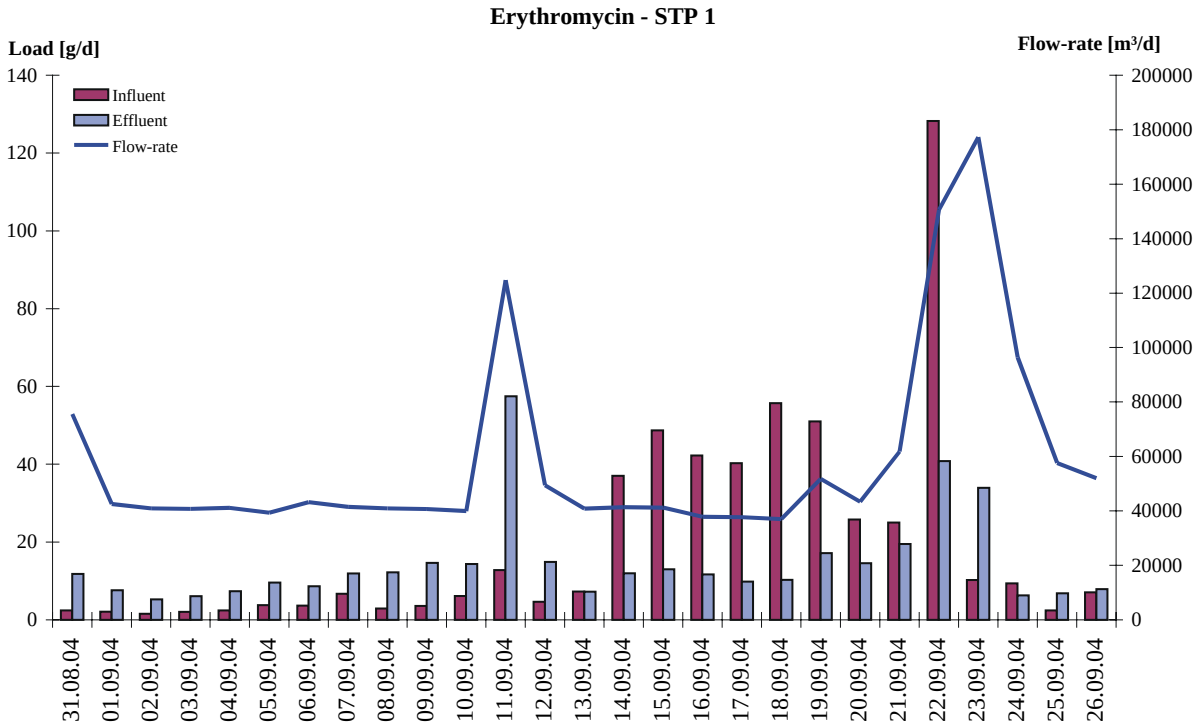


Antibiotics

The daily inflow load of the macrolide antibiotics ranged from 0.9 g up to 60 g during dry weather conditions. During the rain events the loads raises up to 130 g per day. The concentrations ranged from 32 – 1,500 ng/L for erythromycin, 11 - 760 ng/L for clarithromycin and 1.8 – 155 ng/L for roxithromycin in influents. The effluents had maximal concentration of 460 ng/L (ERY), 250 ng/L (CLA) and 126 ng/L (ROX).

As an example for all three macrolide antibiotics the daily loads of erythromycin during the sampling period is shown in Figure 34. During rain events the load of the antibiotics is also raising. This had the same explanation as discussed above for the steroid hormones. However, in the beginning of the sampling period, the load of erythromycin in the effluents were higher than in the corresponding influent. This phenomenon occurs only in this STP. Perhaps consumers of this antibiotic discharged this drug via the toilet and the active substance is released during the treatment process.

Figure 34: Daily loads of erythromycin in the inflow and the effluent of STP 1 over the sampling period. Additionally the wastewater flow-rate during the sampling period is given.



The overall elimination rate of erythromycin in this STP was 23 % while the semi synthetic macrolide antibiotics clarithromycin and roxithromycin were not eliminated. This corresponds to the findings of other authors [29, 98].

4.2.3.2 STP 2

Steroid Hormones

The daily inflow load of the steroid hormones ranged from 0.1 g up to 1.5 g during dry weather conditions, depending on the different types of hormones. For the duration of rainfalls, the wastewater flow-rate raises up from 9,000 m³/d to 32,000 m³/d. Also the inflow load of the steroid hormones raises up to 9 g/d for estriol.

Figure 35: Daily loads of estrone in the inflow and the effluent of STP 2 (trickling filter) over the sampling period. Additionally the wastewater flow-rate during the sampling period is given.

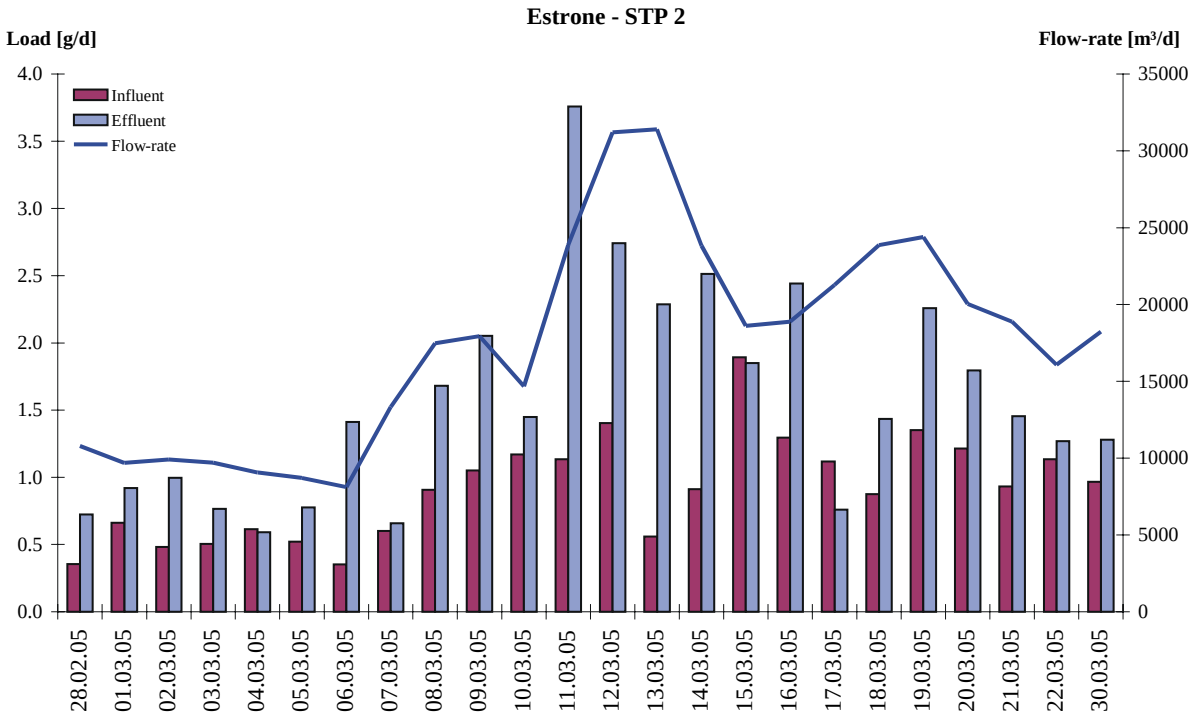
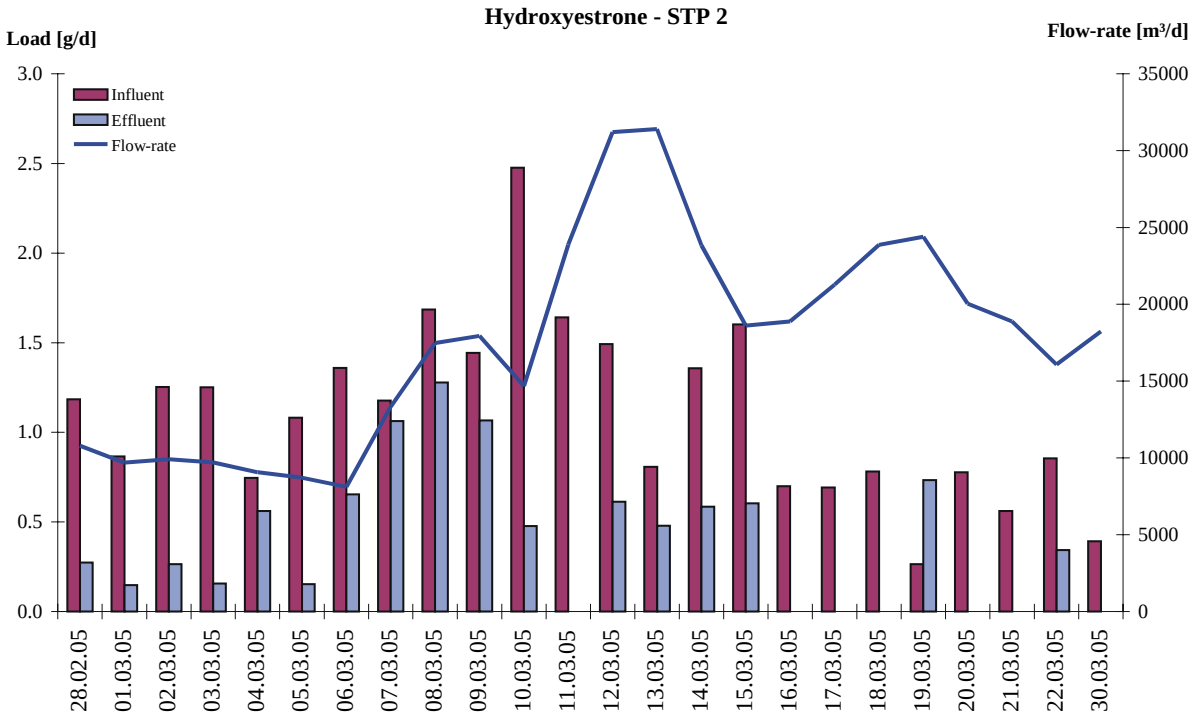


Figure 36: Daily loads of hydroxyestrone in the inflow and the effluent of STP 2 (trickling filter) over the sampling period. Additionally the wastewater flow-rate during the sampling period is presented.



By comparing the loads of the inflow with the load of the effluent, an elimination rate was calculated (Table 32). While the elimination of hydroxyestrone was 60 % (mean value) and those of estriol 34 %, respectively, an elimination of the other hormones was not observed. In contrast an increase of estrone up to 76 % was determined and both hormone sulfate rose about 74 % and 360 %, respectively. This phenomenon could be explained with the assumption that other conjugates like disulfates and sulfate-glucuronides which were not measured, were transformed to β-estradiol 3-sulfate during the wastewater treatment. A transformation of estrone 3-sulfate and other hormone-sulfates to β-estradiol 3-sulfate is possible.

The increase of the steroid hormones based on the transformation of hydroxyestrone to estrone and estradiol is shown in Figure 37. This transformation is described in the literature [99]. The sum of the total inflow load of these three hormones in STP 2 was 54 g in comparison to the sum of the total outflow load 53 g. Thus, no elimination of steroid hormones could be achieved by using a trickling filter for wastewater treatment.

The elimination of 16 α-hydroxyestrone as well as estriol during the complete sampling period also shows a decrease of the elimination efficiency during rainfall. This STP type is also vulnerable for heavy rain events.

Figure 37: Possible transformation route of hydroxyestrone to estrone and estradiol in STP 2

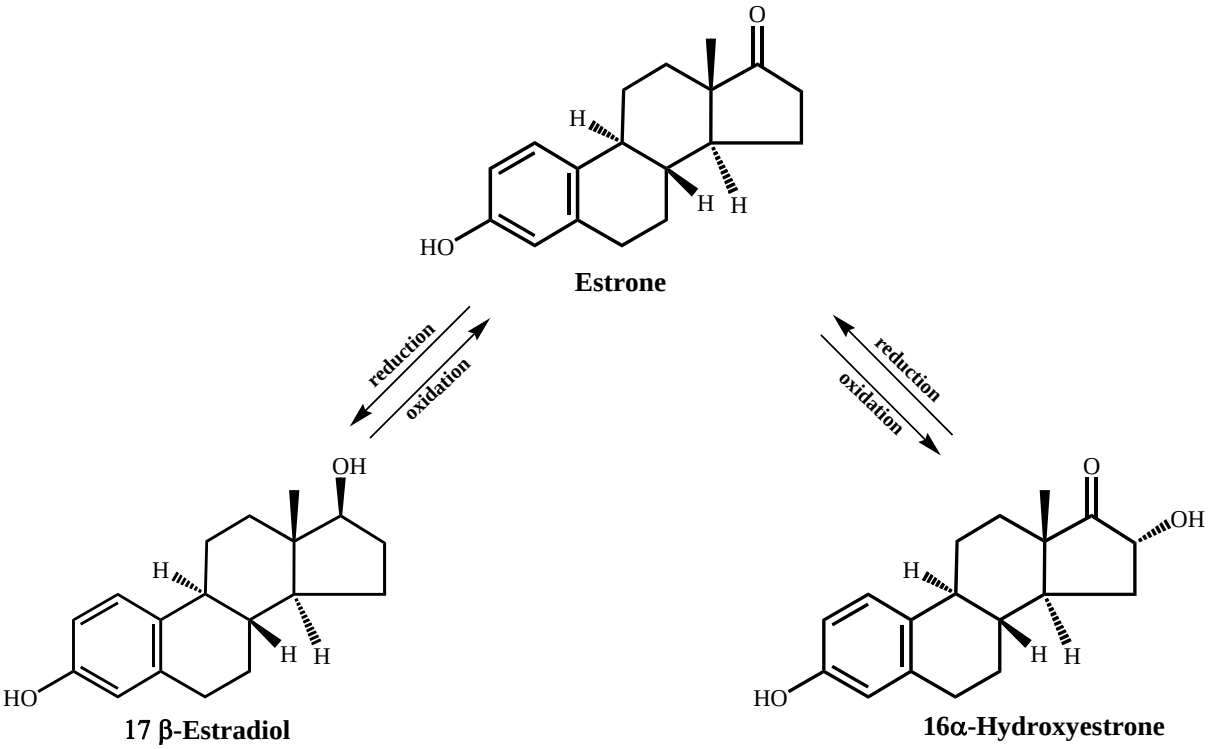
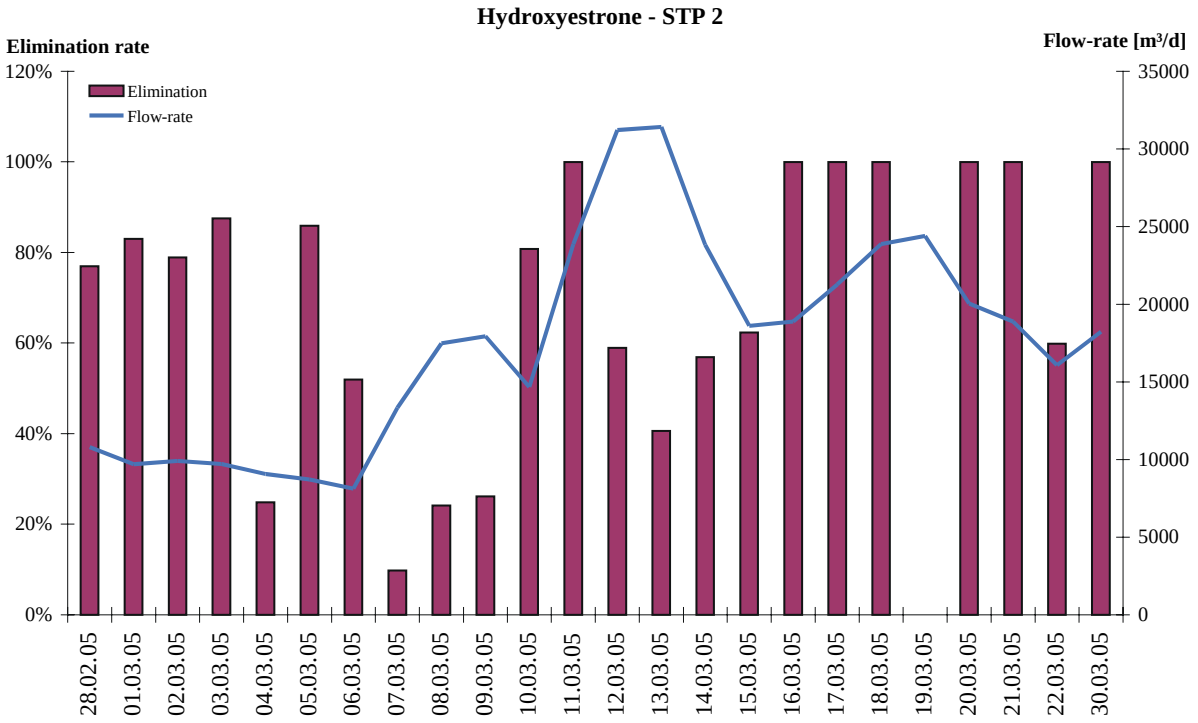


Figure 38: Elimination rates of hydroxyestrone in STP 2 (trickling filter) during the sampling period in comparison to the wastewater flow-rate.

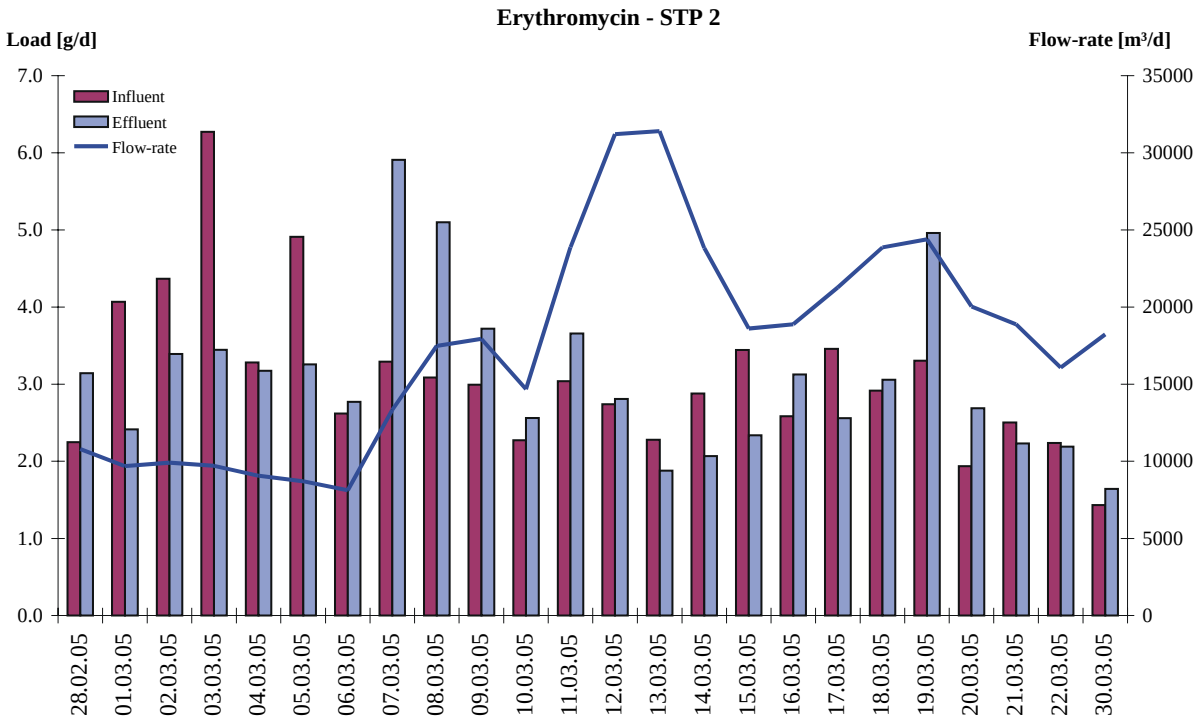


Antibiotics

The daily inflow load of the macrolide antibiotics ranged from 0.3 g up to 7 g. The concentrations ranged from 73 - 650 ng/L for erythromycin, 97 - 690 ng/L for clarithromycin and 16 – 250 ng/L for roxithromycin in influents. The effluents had maximal concentrations of 440 ng/L (ERY), 480 ng/L (CLA) and 350 ng/L (ROX).

As an example for all three macrolide antibiotics, Figure 39 shows the daily loads of erythromycin during the sampling period.

Figure 39: Daily loads of erythromycin as an example for the macrolide antibiotics in the inflow and the effluent of STP 2 (trickling filter) over the sampling period. Additionally the wastewater flow-rate during the sampling period is presented.



The macrolide antibiotics were not eliminated in this STP during the sampling period. Thus, the trickling filter technique does not eliminate macrolide antibiotics during wastewater treatment.

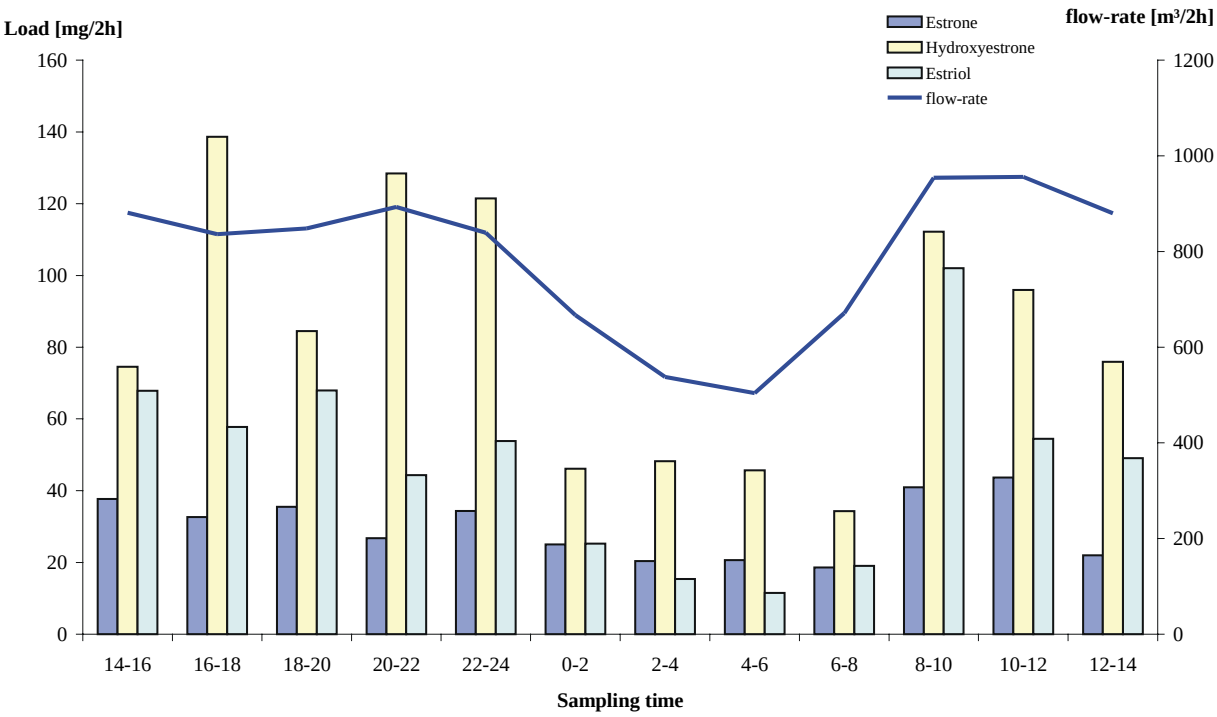
4.2.3.3 STP 3

Steroid Hormones

Figure 40 shows a diurnal cycle of the inflow load of three hormones. Samples were taken in two hour intervals for 24 hours. The loads decreased during nighttime and rose in the morning and evening. While the flow-rate decreased about 50 % during the night, 16 α-hydroxyestrone decreases from 140 mg/2h between 16-18 o'clock to 38 mg/2h between 6-8 o'clock. All other steroid hormones behave similar. This decreasing overnight and increasing in the morning indicates that the steroid hormones stem from excretion by humans. While most people sleep over night, the excretion of hormones via the urine is low. Due to the morning act of urination the excretion of the hormones increases.

Additionally it will be clear why a 24-h flow controlled composite sampling is important for this project. One time sampling of the influent and effluent gives only a snapshot with no information about elimination rates.

Figure 40: Two hours inflow loads of three hormones over one day in STP 3.



The daily inflow load of the steroid hormones in STP 3 ranged from 0.1 g up to 4.2 g during dry weather conditions, depending on the different types of hormones. During rainfalls, the wastewater flow-rate rose from 8,000 m³/d to 35,000 m³/d. Also the inflow load of the steroid hormones rose during this period.

The effluent loads of the final settling tanks were in the same range as the effluent loads of the tertiary ponds. Generally these effluent loads are shifted, because the tertiary ponds holds a volume of 9,600 m³ which is a hydraulic retention time of one day.

As an example for all steroid hormones the daily loads of estrone and hydroxyestrone during the sampling period are presented in Figure 41 and Figure 42.
The concentrations ranged from 14 - 87 ng/L for estrone, 15 – 190 ng/L for hydroxyestrone, up to 18 ng/L for estradiol, 440 ng/L maximal for estriol, up to 26 ng/L for estrone 3-sulfate and <LOD – 28 ng/L for β-estradiol 3-sulfate in influents. The effluents had generally lower concentrations except for β-estradiol 3-sulfate. The maximal concentration of one examined steroid hormone was 140 ng/L in effluents.

Figure 41: Daily loads of estrone in the inflow and the effluent of STP 3 as well as the effluent of the settling tank over the sampling period. Additionally the wastewater flow-rate during the sampling period is presented.

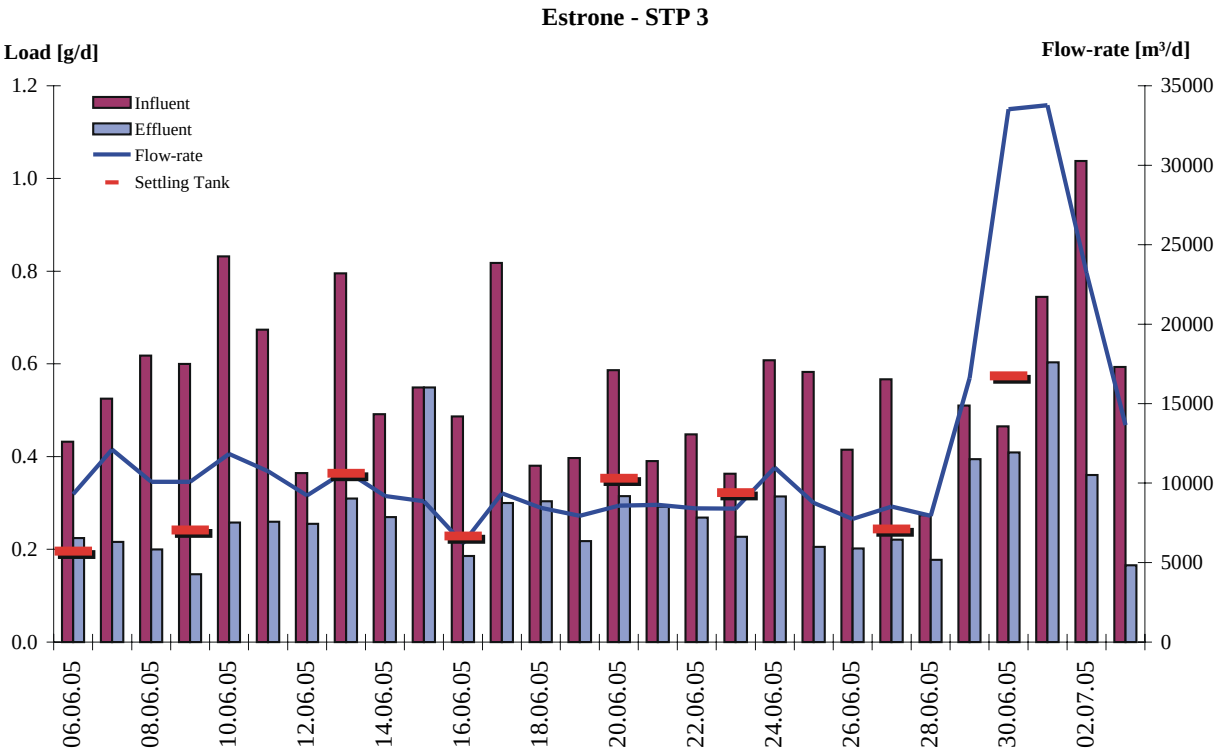
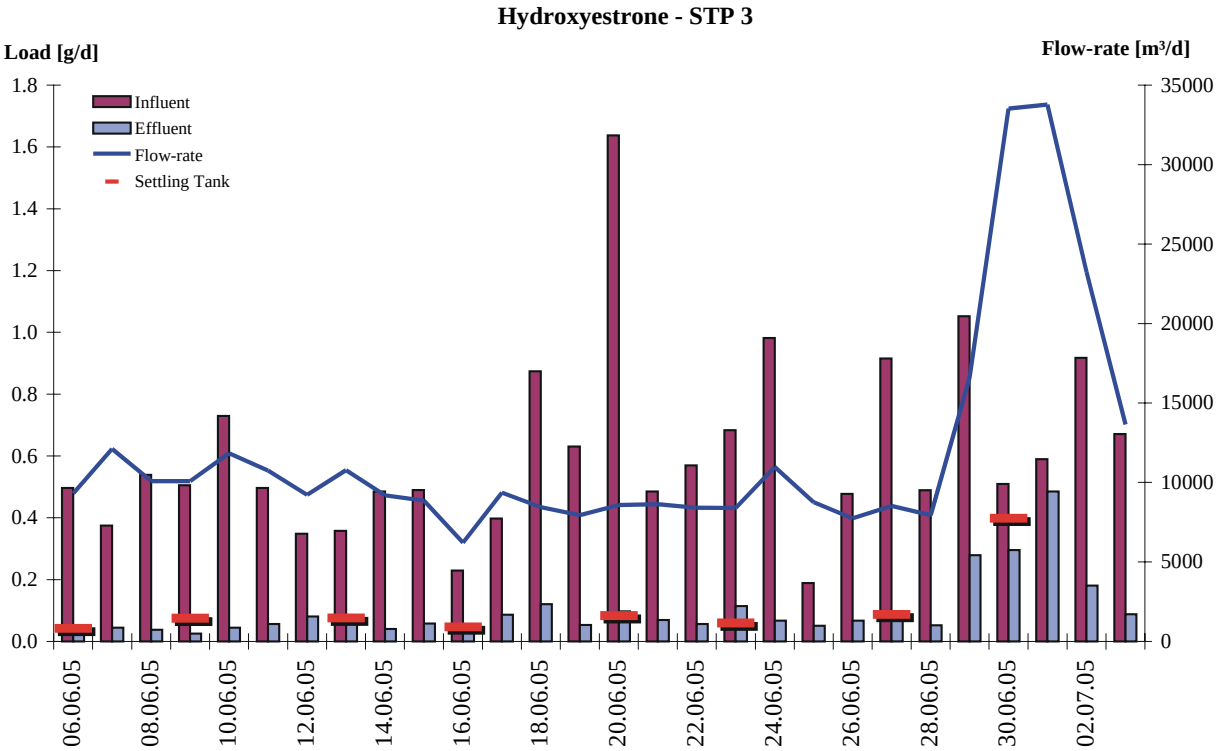


Figure 42: Daily loads of hydroxyestrone in the inflow and the effluent of STP 3 as well as the effluent of the settling tank over the sampling period. Additionally the wastewater flow-rate during the sampling period is presented.



By comparing the loads of the influents with the load of the effluents, an elimination rate was calculated (Table 32). The elimination of the hormones ranged from 30 % – 82 %, except for β-estradiol 3-sulfate. This hormone conjugate shows no significant elimination during wastewater treatment. This could be explained by the assumption that other conjugates like disulfates and sulfate-glucuronides which were not measured, were transformed to β-estradiol 3-sulfate during the wastewater treatment. Also a transformation of estrone 3-sulfate and other hormone-sulfates to β-estradiol 3-sulfate is possible.

The elimination rates of estrone (Figure 43) and hydroxyestrone (Figure 44) as examples for all hormones, except from β-estradiol 3-sulfate, show a dependency on the flow-rate. The sampling in the first 22 days was during a dry weather period, the elimination rates varied extremely from 20% to 80 % for estrone while they were nearly constant for hydroxyestrone (75% to 95 %). Perhaps the STP is too small for a continuous stable elimination of the steroid hormones.

Figure 43: Elimination rates of estrone in STP 3 during the sampling period in comparison to the wastewater flow-rate.

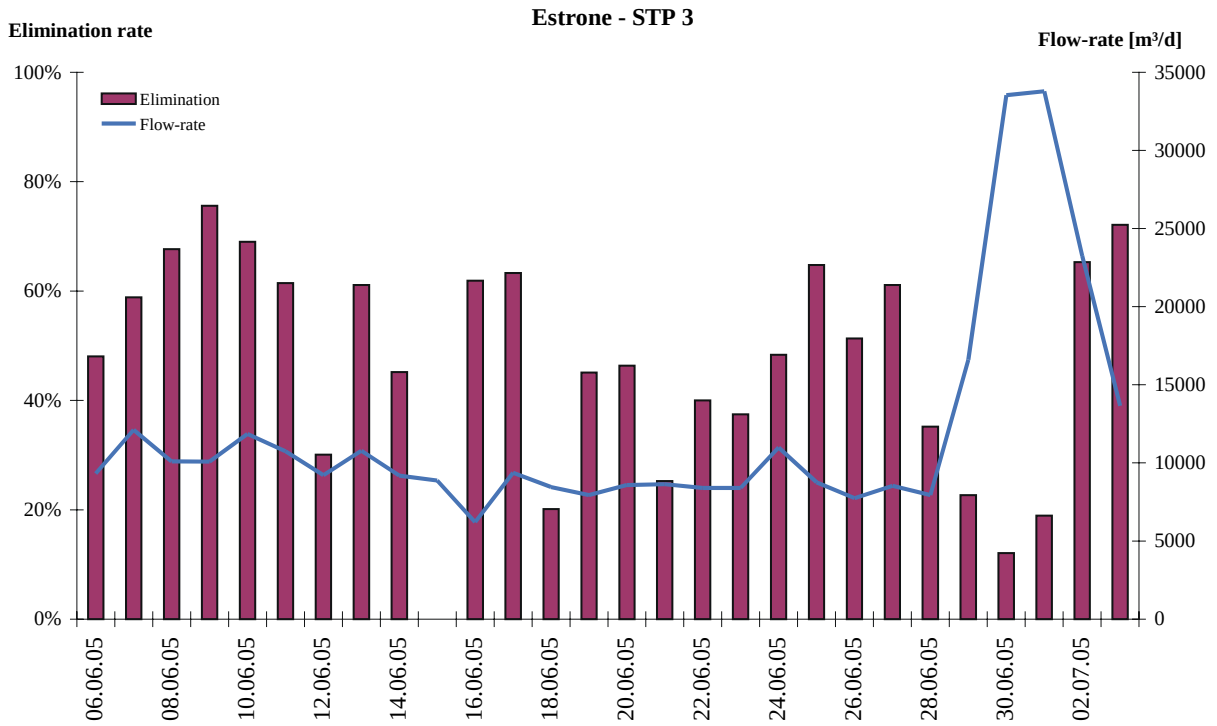
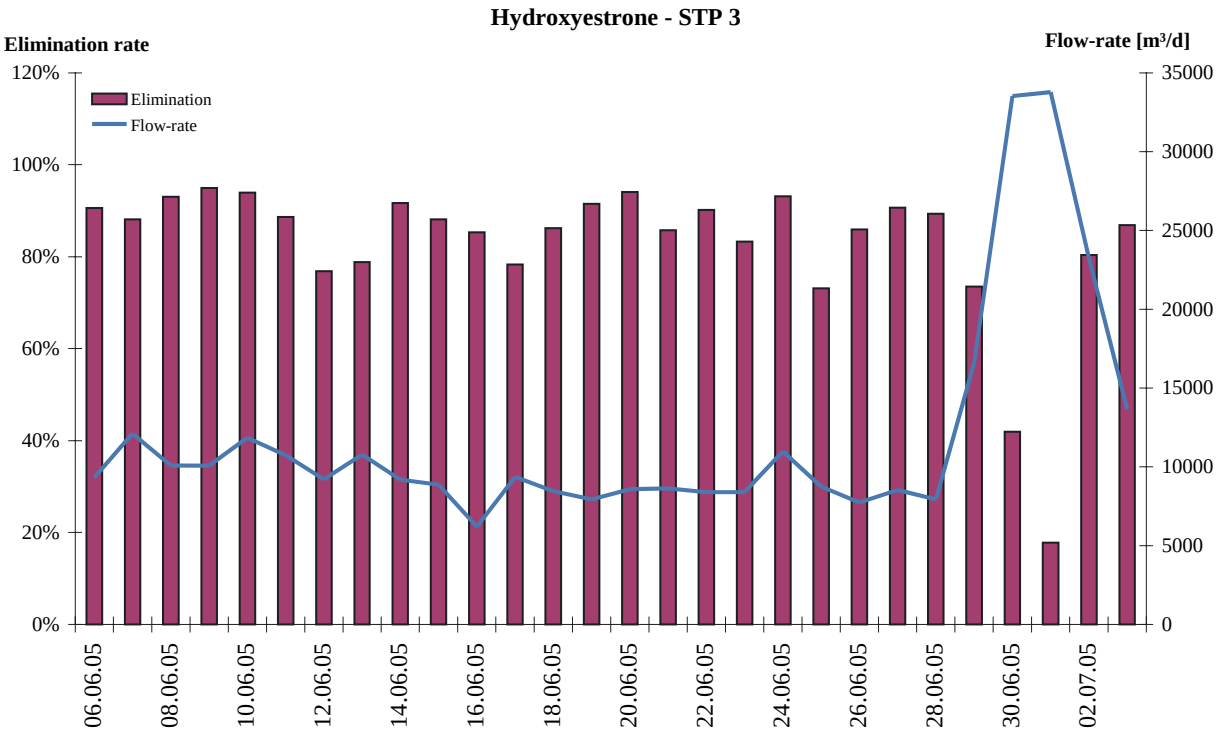


Figure 44: Elimination rates of hydroxyestrone in STP 3 during the sampling period in comparison to the wastewater flow-rate.

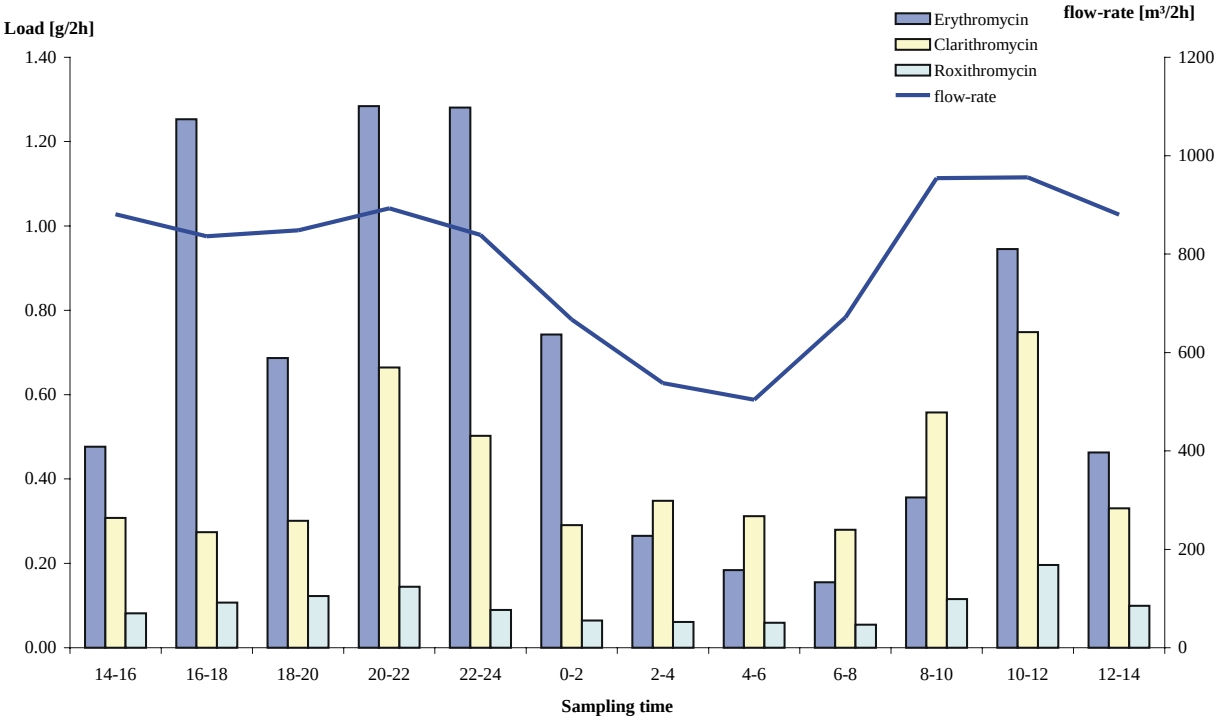


Antibiotics

The 24-h characteristic curve of the antibiotics given in Figure 45 shows a dependency on the time of the day. While the flow-rate decreased about 50 % during the night, erythromycin decreased from 1.3 g/2h between 20-22 o'clock to 0.2 g/2h between 6-8 o'clock. All other macrolide antibiotics behave similar. This decreasing over night and increasing in the morning indicates that also the macrolide antibiotics stems from excretion by humans. The well known 'pee-peak' is also observable.

Additionally, a 24-h flow controlled composite sampling is recommended for the determination of elimination rates for antibiotics.

Figure 45: Two hours inflow load of three hormones over one day in STP 3

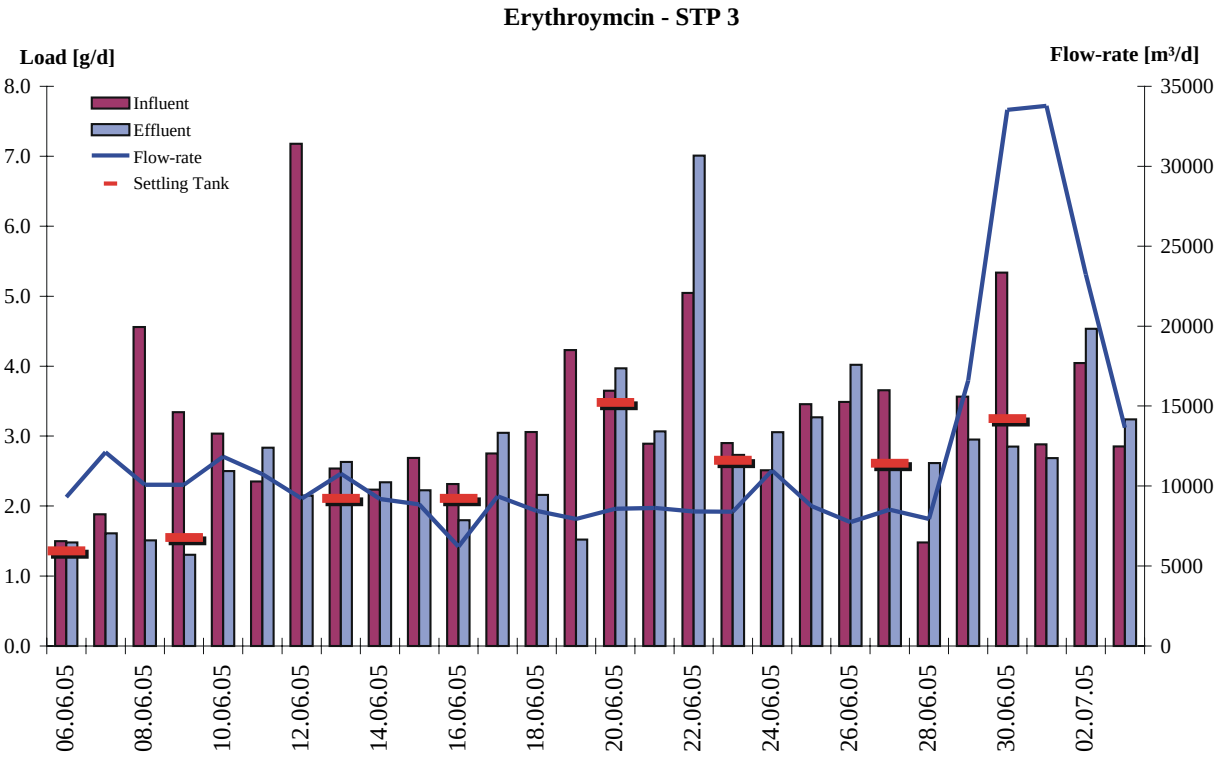


The daily inflow load of the macrolide antibiotics ranged from 0.4 g up to 14 g, while the concentrations ranged from 85 - 780 ng/L for erythromycin, 92 – 1,500 ng/L for clarithromycin and 25 - 94 ng/L for roxithromycin in influents. The effluents had maximal concentration of 830 ng/L (ERY), 520 ng/L (CLA) and 170 ng/L (ROX).

As an example for all three macrolide antibiotics the daily loads of erythromycin during the sampling period are shown in Figure 46.

The macrolide antibiotics were not eliminated significantly in this STP during the sampling period. Thus, a smaller dimension of STPs with a simultaneous denitrification step does not help to eliminate macrolide antibiotics during wastewater treatment.

Figure 46: Daily loads of erythromycin as an example for the macrolide antibiotics n the inflow and the effluent of STP 3 as well as the effluent of the settling tank over the sampling period. Additionally the wastewater flow-rate during the sampling period is presented.



4.2.3.4 Comparison of the three STPs

By comparison of all investigated STPs, the differences between STP 2 and STP 1 are especially remarkable. STP 2 is equipped with a trickling filter and serves only 64,000 inhabitant equivalent values (IEV) while the more modern STP 1 handles 250,000 IEV (Table 33). This comparison showed that the amount of steroid hormones released into the environment per day is up to a factor 6 higher in the smaller STP 2 than in the activated sludge plant (STP 1).

No significant elimination for the macrolide antibiotics could be detected in any of the three STPs of this project. The daily discharge of these compounds raises up as expected with the number of affiliated persons (Table 33).

Table 33: Comparison of the daily discharge of steroid hormones and macrolide antibiotics in the three investigated STPs and inhabitant equivalent values (IEV)

	STP 1 (250.000) IEV	**STP 2 (64.000) IEV**	**STP 3 (32.000) IEV**
	Discharge [mg/d]	**Discharge [mg/d]**	**Discharge [mg/d]**
Estrone	253	1578	280
16 α-Hydroxyestrone	414	394	111
17β-Estradiol	340	288	84
Estriol	2098	3215	358
Estrone 3-sulfate	271	764	28
β-Estradiol 3-sulfate	512	393	268
Erythromycin	14555	3087	2750
Clarithromycin	8770	4419	3679
Roxithromycin	4618	1917	1282

4.2.4 Conclusions to the fate of hormones and antibiotics in wastewater

It has been demonstrated that steroid hormones and macrolide antibiotics were released into the environment via the pathway human, urine, wastewater, sewage treatment plant and effluents of STPs. Three different concepts of STPs were investigated to determine their elimination rates.

Larger STPs eliminate hormones more constantly than small STPs. Heavy rainfall events, which resulted in high wastewater flow-rates, led to a collapse of the biological treatment concerning these compounds. More rain storage basins are necessary to reduce these influence on the wastewater treatment process.

By means of the trickling filter technique the steroid hormones could not be eliminate during the wastewater treatment. Only a transformation of the hormones among each other was observed. This technique should be replaced with more state of the art treatment techniques.

No significant elimination for the macrolide antibiotics could be detected in all three STPs. New concepts of treatment should be developed for the elimination of macrolide antibiotics during wastewater treatment if environmental issues were taken into consideration.

5 Overall Conclusions

It has been demonstrated that two pathways of pharmaceuticals are prevalent in the environment. Path A starts with the application of human medical drugs, the excretion of drugs and transport via the sewer to sewage treatment plants. Finally the elimination of the pharmaceuticals in sewage treatment plants and discharging the treated water into the environment. A precise and robust analytical method in compliance within EU decision 657/2002/EC was established for the determination of macrolide antibiotics, steroid hormones, hormone conjugates and oral contraceptives in heavily matrix loaded water. The investigation of the elimination efficiency of municipal sewage treatment plants has shown, that there is a release of high amounts of antibiotics and hormones into the environment. The elimination efficiency is also dependent on the actual weather condition. Heavy rainfall events can reduce the elimination of pharmaceuticals in a STP. It was also demonstrated that old treatment techniques like trickling filters were not able to eliminate these ‘new’ pollutions.

The second path describes the application of veterinary drugs used for the prevention and treatment of infections in animal husbandry. The applied pharmaceuticals are excreted and transferred with the manure to manure tanks. During the storage of manure for several months the excreted pharmaceuticals undergo diverse transformation and degradation processes or remain unchanged in the manure. In a degradation experiment it was demonstrated that tiamulin persists in liquid manure while salinomycin was degraded rapidly. Several metabolites of salinomycin were identified during this manure degradation experiment. Two times in a year, fields are fertilised with liquid manure. The soil will be contaminated with the pharmaceuticals that are contained in the manure. It was demonstrated that soil, taken from fields which were fertilised with liquid manure containing antibiotics, had high amounts of antibiotics in the surface layer, as well as several months after fertilising. A degradation experiment of antibiotics in soil with similar aerobic field conditions affirmed these field sampling results that antibiotics persist in the environment.

This work contributed significantly to the understanding of the environmental occurrence and fate of antibiotics and estrogenic hormones.

6 Used equipment and analytical standards

6.1 Equipment

6.1.1 Mass Spectrometer

TSQ 7000 with APCI-2 and ESI-2 ion source (Finnigan MAT, Bremen, Germany)
API 2000 with Heated Nebulizer and TurboIon Spray (Applied Biosystems, Darmstadt, Germany)
Bio TOF III with ESI multispray ion source (Bruker, Bremen, Germany)

6.1.2 HPLC

6.1.2.1 Pump

P 580 HPG binary high pressure gradient pump (Dionex, Idstein, Germany)
LC-10 AT isocratic pump (Shimadzu, Duisburg, Germany)
G1312A binary high pressure gradient pump with solvent selector (Agilent, Waldbronn, Germany)
G1312A binary high pressure gradient pump (Agilent, Waldbronn, Germany)
G1311A quaternary low pressure gradient pump (Agilent, Waldbronn, Germany)

6.1.2.2 Autosampler

Gina 50 (Dionex, Idstein, Germany)
G1313A (Agilent, Waldbronn, Germany)

6.1.2.3 Degasser

DG-1210 (Dionex, Idstein, Germany)
G1379A (Agilent, Waldbronn, Germany)
G1322A (Agilent, Waldbronn, Germany)

6.1.2.4 Column Oven

STH (Dionex, Idstein, Germany)

G1316A with column selector (Agilent, Waldbronn, Germany)

6.1.2.5 HPLC Column

Phenosphere Next 3 µm, C18 (2) 150 x 2 mm (Phenomenex, Torrance CA, United States)

Synergi RP-Max 4 µm, 150 x 2 mm (Phenomenex, Torrance CA, United States)

Phenogel SEC column 5 µm, 100 Å, 21.2 x 300 mm (Phenomenex, Torrance CA, United States)

6.1.3 Valve

C2-2006D automatic valco valve (VICI AG, Schenkon, Switzerland)

6.1.4 Evaporator

Laborota 4001 with Rotavac Control (Heidolph, Kelheim, Germany)

Büchi Syncore® Analyst 12 port evaporation unit (Büchi, Essen, Germany)

6.1.5 Extractor

ASE® 200 (Dionex, Idstein, Germany)

6.1.6 Software

Chromeleon Vers. 6.00 (Dionex, Idstein, Germany)

ICL Vers. 8.3.2 (Thermo Electron Corporation, West Palm Beach FL, United States)

Xcalibur™ Vers. 1.2 (Thermo Electron Corporation, West Palm Beach FL, United States)

Xcalibur™ Vers. 1.3 (Thermo Electron Corporation, West Palm Beach FL, United States)

Analyst 1.4 (Applied Biosystems, Darmstadt, Germany)

6.2 Analytical Standards

Clarithromycin (Promochem, Wesel, Germany).

Erythromycin, 98% (Sigma-Aldrich, Seelze, Germany)

17β-Estradiol hemihydrate, Vetranal™, (Riedel-de Haën, Seelze, Germany)

β-Estradiol 17-acetate, Vetranal™, (Riedel-de Haën, Seelze, Germany)

β-Estradiol 3-sulfate sodium salt, (Sigma-Aldrich, Seelze, Germany)

Estriol, (Sigma-Aldrich, Seelze, Germany)

Estrone, (Sigma-Aldrich, Seelze, Germany)

Estrone 2,4,16,16 - d_4, (Sigma-Aldrich, Seelze, Germany)

Estrone 3-sulfate potassium salt, (Sigma-Aldrich, Seelze, Germany)

Estrone 3-sulfate 2,4,16,16 - d_4 sodium salt (Dr. Ehrenstorfer GmbH, Augsburg, Germany)

17α-Ethinylestradiol, Vetranal™, (Riedel-de Haën, Seelze, Germany)

17α-Ethinylestradiol 2,4,16,16 - d_4 (Dr. Ehrenstorfer GmbH, Augsburg, Germany)

16α-Hydroxyestrone, (Sigma-Aldrich, Seelze, Germany)

Ivermectin, main compound B_{1a} (Sigma-Aldrich, Seelze, Germany)

Roxithromycin, 90 % HPLC, (Sigma-Aldrich, Seelze, Germany)

Salinomycin SV sodium salt, 83.2 % HPLC, , Vetranal™, (Riedel-de Haën, Seelze, Germany)

Mestranol, Vetranal™, (Riedel-de Haën, Seelze, Germany)

Monensin sodium salt, 89.6 % HPLC, , Vetranal™, (Riedel-de Haën, Seelze, Germany)

Oleandomycinphosphatedihydrate, 86.2 % HPLC, Vetranal™, (Riedel-de Haën, Seelze, Germany)

Tiamulinfumarate, 99.9 % HPLC, Vetranal™, (Riedel-de Haën, Seelze, Germany)

Tylosintartrate, 918 µg Tylosin base per 1 mg Tylosintatrat, (Sigma-Aldrich, Seelze, Germany)

Solvents were used as described in the respective chapters.

7 Acknowledgment

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Jens Andresen for proofreading again.

The assistance help of Jennifer Hardes during the time-consuming SEC-cleanup of the BASPiK project.

The skilled laboratory work of the students Tobias Friedrich and Monika Dreier and the laboratory technicians of the Institute of Environmental Research Gabriele Hardes, Cornelia Stolle and Silke Richter is also acknowledged.

Christof A. Michalski for transporting the wastewater samples and for special entertainment issues.

The cheerful “Doppelkopf” sessions with Jochen Türk, Klaus Silex, Jörn Sickerling, Jens Andresen, formerly known as Jens Meyer, and Kai Bester, where many problems have been solved.

And my parents for financial support during this PhD work and the daily invitations to dinner.

8 Index

8.1 Tables

8.2 Figures

9 Literature

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10 Supplement

Table I: Date of sampling, flow rate and influent and effluent concentration and load of erythromycin in STP 1.

STP 1 Erythromycin					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	32	160	2.4	12
01.09.04	42585	49	180	2.1	7.6
02.09.04	40910	39	130	1.6	5.3
03.09.04	40751	51	150	2.1	6.1
04.09.04	41171	58	180	2.4	7.4
05.09.04	39362	95	240	3.8	9.6
06.09.04	43254	85	200	3.7	8.7
07.09.04	41523	160	290	6.7	12
08.09.04	40899	71	300	2.9	12
09.09.04	40697	87	360	3.5	15
10.09.04	39940	150	360	6.1	14
11.09.04	124737	100	460	13	57
12.09.04	49420	94	300	4.7	15
13.09.04	40835	180	180	7.3	7.2
14.09.04	41371	37	290	1.5	12
15.09.04	41231	1200	320	49	13
16.09.04	37846	1100	310	42	12
17.09.04	37722	1100	260	40	9.8
18.09.04	37019	1500	280	56	10
19.09.04	51808	990	330	51	17
20.09.04	43370	600	340	26	15
21.09.04	61798	400	320	25	20
22.09.04	150538	850	270	128	41
23.09.04	177322	58	190	10	34
24.09.04	96396	97	66	9.4	6.3
25.09.04	57588	42	120	2.4	6.8
26.09.04	52028	140	150	7.1	7.9

Table II: Date of sampling, flow rate and influent and effluent concentration and load of clarithromycin in STP 1.

STP 1 Clarithromycin					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	20	83	1.5	6.2
01.09.04	42585	32	99	1.4	4.2
02.09.04	40910	26	74	1.1	3.0
03.09.04	40751	40	89	1.6	3.6
04.09.04	41171	62	100	2.6	4.2
05.09.04	39362	100	110	4.0	4.4
06.09.04	43254	77	120	3.3	5.0
07.09.04	41523	78	140	3.2	5.9
08.09.04	40899	76	140	3.1	5.7
09.09.04	40697	67	160	2.7	6.6
10.09.04	39940	87	190	3.5	7.7
11.09.04	124737	110	200	14	24
12.09.04	49420	58	170	2.9	8.1
13.09.04	40835	150	110	5.9	4.3
14.09.04	41371	11	180	0.4	7.4
15.09.04	41231	300	190	12	7.8
16.09.04	37846	260	190	9.8	7.2
17.09.04	37722	760	220	29	8.4
18.09.04	37019	360	250	13	9.1
19.09.04	51808	200	240	10	12
20.09.04	43370	420	250	18	11
21.09.04	61798	250	240	15	15
22.09.04	150538	170	180	26	27
23.09.04	177322	39	110	6.9	19
24.09.04	96396	67	67	6.5	6.5
25.09.04	57588	51	110	2.9	6.1
26.09.04	52028	110	130	5.9	6.6

Table III: Date of sampling, flow rate and influent and effluent concentration and load of roxithromycin in STP 1.

STP 1 Roxithromycin					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	12	52	0.9	3.9
01.09.04	42585	25	65	1.1	2.8
02.09.04	40910	16	50	0.6	2.0
03.09.04	40751	22	60	0.9	2.4
04.09.04	41171	51	66	2.1	2.7
05.09.04	39362	57	78	2.2	3.1
06.09.04	43254	74	79	3.2	3.4
07.09.04	41523	47	90	2.0	3.7
08.09.04	40899	26	91	1.0	3.7
09.09.04	40697	17	90	0.7	3.6
10.09.04	39940	51	92	2.0	3.7
11.09.04	124737	83	89	10	11
12.09.04	49420	29	75	1.4	3.7
13.09.04	40835	71	47	2.9	1.9
14.09.04	41371	120	73	5.0	3.0
15.09.04	41231	120	81	5.0	3.3
16.09.04	37846	140	89	5.2	3.4
17.09.04	37722	120	120	4.6	4.3
18.09.04	37019	160	110	5.7	4.0
19.09.04	51808	150	120	7.6	6.1
20.09.04	43370	95	130	4.1	5.5
21.09.04	61798	130	130	7.9	7.7
22.09.04	150538	82	99	12	15
23.09.04	177322	30	60	5.3	11
24.09.04	96396	41	29	3.9	2.8
25.09.04	57588	54	55	3.1	3.1
26.09.04	52028	67	74	3.5	3.8

Table IV: Date of sampling, flow rate and influent and effluent concentration and load of β-estradiol 3-sulfate in STP 1.

STP 1 β-Estradiol 3-sulfate					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	8.5-28*	7.1	2.1	0.5
01.09.04	42585	<LOD	0.6-1.8*	0.4	0.1
02.09.04	40910	<LOD	3.4	0.3	0.1
03.09.04	40751	<LOD	0.6-1.8*	0.3	0.1
04.09.04	41171	<LOD	5.6	0.3	0.2
05.09.04	39362	<LOD	0.6-1.8*	0.3	0.1
06.09.04	43254	<LOD	0.6-1.8*	0.4	0.1
07.09.04	41523	<LOD	7.2	0.4	0.3
08.09.04	40899	<LOD	0.6-1.8*	0.3	0.1
09.09.04	40697	8.5-28*	14.0	1.1	0.6
10.09.04	39940	8.5-28*	0.6-1.8*	1.1	0.1
11.09.04	124737	<LOD	0.6-1.8*	1.1	0.2
12.09.04	49420	8.5-28*	7.1	1.4	0.4
13.09.04	40835	<LOD	4.7	0.3	0.2
14.09.04	41371	<LOD	3.5	0.4	0.1
15.09.04	41231	8.5-28*	6.5	1.2	0.3
16.09.04	37846	8.5-28*	36	1.1	1.4
17.09.04	37722	8.5-28*	3.1	1.1	0.1
18.09.04	37019	8.5-28*	15	1.0	0.5
19.09.04	51808	8.5-28*	9.4	1.5	0.5
20.09.04	43370	8.5-28*	16	1.2	0.7
21.09.04	61798	8.5-28*	<LOD	1.7	0.0
22.09.04	150538	<LOD	3.0	1.3	0.5
23.09.04	177322	<LOD	3.6	1.5	0.6
24.09.04	96396	<LOD	37	0.8	3.6
25.09.04	57588	<LOD	12	0.5	0.7
26.09.04	52028	<LOD	35	0.4	1.8

Table V: Date of sampling, flow rate and influent and effluent concentration and load of estrone 3-sulfate in STP 1.

STP 1 Estrone 3-sulfate					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	5.5	1.2	0.4	0.1
01.09.04	42585	1.2-4*	1.0	0.2	0.0
02.09.04	40910	1.2-4*	0.9	0.2	0.0
03.09.04	40751	<LOD	0.7	0.0	0.0
04.09.04	41171	<LOD	1.1	0.0	0.0
05.09.04	39362	<LOD	1.5	0.0	0.1
06.09.04	43254	<LOD	0.7	0.1	0.0
07.09.04	41523	1.2-4*	5.1	0.0	0.2
08.09.04	40899	<LOD	8.3	0.0	0.3
09.09.04	40697	4.2	7.5	0.2	0.3
10.09.04	39940	10	1.2	0.4	0.0
11.09.04	124737	1.2-4*	<LOD	0.5	0.1
12.09.04	49420	1.2-4*	2.7	0.2	0.1
13.09.04	40835	1.2-4*	4.5	0.2	0.2
14.09.04	41371	<LOD	12	0.0	0.5
15.09.04	41231	12	7.8	0.5	0.3
16.09.04	37846	<LOD	12	0.0	0.5
17.09.04	37722	8.1	13	0.3	0.5
18.09.04	37019	17	12	0.6	0.5
19.09.04	51808	9.3	9.4	0.5	0.5
20.09.04	43370	10	<LOD	0.4	0.0
21.09.04	61798	10	9.7	0.6	0.6
22.09.04	150538	7.3	1.3	1.1	0.2
23.09.04	177322	<LOD	3.1	0.2	0.5
24.09.04	96396	8.4	7.8	0.8	0.8
25.09.04	57588	1.2-4*	9.8	0.2	0.6
26.09.04	52028	10	6.1	0.5	0.3

Table VI: Date of sampling, flow rate and influent and effluent concentration and load of estriol in STP 1.

STP 1 Estriol					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	101	45	7.7	3.4
01.09.04	42585	12-35*	97	1.5	4.1
02.09.04	40910	220	160	8.8	6.7
03.09.04	40751	12-35*	4.5-15*	1.4	0.6
04.09.04	41171	<LOD	120	0.5	5.0
05.09.04	39362	12-35*	4.5-15*	1.4	0.6
06.09.04	43254	44	4.5-15*	1.9	0.6
07.09.04	41523	89	37	3.7	1.5
08.09.04	40899	36	4.5-15*	1.5	0.6
09.09.04	40697	12-35*	4.5-15*	1.4	0.6
10.09.04	39940	84	4.5-15*	3.4	0.6
11.09.04	124737	12-35*	32	4.4	4.0
12.09.04	49420	124	82	6.1	4.1
13.09.04	40835	12-35*	4.5-15*	1.4	0.6
14.09.04	41371	<LOD	<LOD	0.5	0.2
15.09.04	41231	470	99	19	4.1
16.09.04	37846	510	130	19	4.8
17.09.04	37722	150	<LOD	5.8	0.2
18.09.04	37019	200	66	7.5	2.4
19.09.04	51808	68	4.5-15*	3.5	0.8
20.09.04	43370	67	4.5-15*	2.9	0.7
21.09.04	61798	130	76	8.0	4.7
22.09.04	150538	66	<LOD	9.9	0.7
23.09.04	177322	12-35*	<LOD	6.2	0.8
24.09.04	96396	12-35*	<LOD	3.4	0.4
25.09.04	57588	12-35*	<LOD	2.0	0.3
26.09.04	52028	12-35*	70	1.8	3.6

Table VII: Date of sampling, flow rate and influent and effluent concentration and load of 16a-hydroxyestrone in STP 1.

STP 1 16α-Hydroxyestrone					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	15	<LOD	1.1	0.2
01.09.04	42585	16	<LOD	0.7	0.1
02.09.04	40910	17	<LOD	0.7	0.1
03.09.04	40751	19	<LOD	0.8	0.1
04.09.04	41171	21	<LOD	0.8	0.1
05.09.04	39362	26	<LOD	1.0	0.1
06.09.04	43254	13	<LOD	0.6	0.1
07.09.04	41523	18	<LOD	0.7	0.1
08.09.04	40899	14	<LOD	0.6	0.1
09.09.04	40697	20	<LOD	0.8	0.1
10.09.04	39940	11	<LOD	0.5	0.1
11.09.04	124737	14	<LOD	1.7	0.3
12.09.04	49420	23	<LOD	1.1	0.1
13.09.04	40835	13	2.4-8*	0.5	0.3
14.09.04	41371	2.4-8*	<LOD	0.3	0.1
15.09.04	41231	95	2.4-8*	3.9	0.3
16.09.04	37846	73	14	2.8	0.5
17.09.04	37722	105	17	3.9	0.6
18.09.04	37019	32	12	1.2	0.4
19.09.04	51808	35	2.4-8*	1.8	0.4
20.09.04	43370	53	2.4-8*	2.3	0.3
21.09.04	61798	29	2.4-8*	1.8	0.5
22.09.04	150538	19	14	2.9	2.1
23.09.04	177322	11	14	2.0	2.5
24.09.04	96396	2.4-8*	2.4-8*	0.8	0.8
25.09.04	57588	2.4-8*	2.4-8*	0.5	0.5
26.09.04	52028	<LOD	<LOD	0.1	0.1

Table VIII: Date of sampling, flow rate and influent and effluent concentration and load of 17β-estradiol in STP 1.

STP 1 17β-Estradiol					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	46	<LOD	3.5	0.2
01.09.04	42585	40	<LOD	1.7	0.1
02.09.04	40910	43	<LOD	1.8	0.1
03.09.04	40751	42	<LOD	1.7	0.1
04.09.04	41171	52	<LOD	2.1	0.1
05.09.04	39362	68	<LOD	2.7	0.1
06.09.04	43254	41	<LOD	1.8	0.1
07.09.04	41523	27	2.4-8*	1.1	0.3
08.09.04	40899	14	<LOD	0.6	0.1
09.09.04	40697	22	<LOD	0.9	0.1
10.09.04	39940	13	<LOD	0.5	0.1
11.09.04	124737	17	<LOD	2.2	0.3
12.09.04	49420	9.2	<LOD	0.5	0.1
13.09.04	40835	17	<LOD	0.7	0.1
14.09.04	41371	<LOD	<LOD	0.1	0.1
15.09.04	41231	12	<LOD	0.5	0.1
16.09.04	37846	17	8.4	0.7	0.3
17.09.04	37722	23	12	0.9	0.5
18.09.04	37019	34	2.4-8*	1.3	0.3
19.09.04	51808	40	2.4-8*	2.1	0.4
20.09.04	43370	36	2.4-8*	1.6	0.3
21.09.04	61798	16	8.9	1.0	0.5
22.09.04	150538	11	8.4	1.6	1.3
23.09.04	177322	12	2.4-8*	2.2	1.4
24.09.04	96396	22	2.4-8*	2.1	0.8
25.09.04	57588	22	19	1.3	1.1
26.09.04	52028	<LOD	<LOD	0.1	0.1

Table IX: Date of sampling, flow rate and influent and effluent concentration and load of estrone in STP 1.

STP 1 Estrone					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
31.08.04	75579	80	7.3	6.1	0.6
01.09.04	42585	45	3.4	1.9	0.1
02.09.04	40910	83	4.3	3.4	0.2
03.09.04	40751	61	3.7	2.5	0.2
04.09.04	41171	47	3.1	1.9	0.1
05.09.04	39362	65	2.1	2.6	0.1
06.09.04	43254	60	<LOD	2.6	0.0
07.09.04	41523	82	4.6	3.4	0.2
08.09.04	40899	44	0.6-2*	1.8	0.1
09.09.04	40697	73	3.5	3.0	0.1
10.09.04	39940	57	2.8	2.3	0.1
11.09.04	124737	55	0.6-2*	6.8	0.2
12.09.04	49420	40	<LOD	2.0	0.0
13.09.04	40835	47	4.9	1.9	0.2
14.09.04	41371	70	<LOD	2.9	0.0
15.09.04	41231	92	<LOD	3.8	0.0
16.09.04	37846	59	<LOD	2.2	0.0
17.09.04	37722	130	<LOD	4.9	0.0
18.09.04	37019	38	<LOD	1.4	0.0
19.09.04	51808	37	<LOD	1.9	0.0
20.09.04	43370	57	<LOD	2.5	0.0
21.09.04	61798	67	<LOD	4.2	0.0
22.09.04	150538	45	<LOD	6.7	0.0
23.09.04	177322	19	<LOD	3.3	0.0
24.09.04	96396	34	0.6-2*	3.3	0.1
25.09.04	57588	38	58	2.2	0.0
26.09.04	52028	51	13	2.6	0.7

Table X: Date of sampling, flow rate and influent and effluent concentration and load of erythromycin in STP 2.

STP 2 Erythromycin					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	210	290	2.2	3.1
01.03.05	9695	420	250	4.1	2.4
02.03.05	9908	440	340	4.4	3.4
03.03.05	9708	650	360	6.3	3.4
04.03.05	9062	360	350	3.3	3.2
05.03.05	8713	560	370	4.9	3.3
06.03.05	8118	320	340	2.6	2.8
07.03.05	13332	250	440	3.3	5.9
08.03.05	17474	180	290	3.1	5.1
09.03.05	17943	170	210	3.0	3.7
10.03.05	14675	160	180	2.3	2.6
11.03.05	23874	130	150	3.0	3.7
12.03.05	31214	88	90	2.7	2.8
13.03.05	31413	73	60	2.3	1.9
14.03.05	23829	120	87	2.9	2.1
15.03.05	18610	190	130	3.4	2.3
16.03.05	18880	140	170	2.6	3.1
17.03.05	21269	160	120	3.5	2.6
18.03.05	23865	120	130	2.9	3.1
19.03.05	24391	140	200	3.3	5.0
20.03.05	20040	97	130	1.9	2.7
21.03.05	18890	130	120	2.5	2.2
22.03.05	16082	104	140	2.2	2.2
30.03.05	18225	79	90	1.4	1.6

Table XI: Date of sampling, flow rate and influent and effluent concentration and load of clarithromycin in STP 2.

STP 2 Clarithromycin					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	330	390	3.6	4.3
01.03.05	9695	690	390	6.7	3.8
02.03.05	9908	490	460	4.8	4.6
03.03.05	9708	440	440	4.3	4.3
04.03.05	9062	500	400	4.6	3.6
05.03.05	8713	400	400	3.6	3.5
06.03.05	8118	450	450	3.6	3.7
07.03.05	13332	370	480	4.9	6.4
08.03.05	17474	310	380	5.4	6.6
09.03.05	17943	330	310	5.9	5.6
10.03.05	14675	420	290	6.2	4.2
11.03.05	23874	170	320	4.2	7.7
12.03.05	31214	130	170	4.1	5.4
13.03.05	31413	140	120	4.4	3.7
14.03.05	23829	190	160	4.5	3.7
15.03.05	18610	220	210	4.1	3.9
16.03.05	18880	180	210	3.4	4.0
17.03.05	21269	170	160	3.6	3.4
18.03.05	23865	140	150	3.3	3.4
19.03.05	24391	160	300	3.8	7.4
20.03.05	20040	190	180	3.8	3.5
21.03.05	18890	280	200	5.2	3.7
22.03.05	16082	190	240	3.1	3.9
30.03.05	18225	97	100	1.8	1.9

Table XII: Date of sampling, flow rate and influent and effluent concentration and load of roxithromycin in STP 2.

STP 2 Roxithromycin					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	160	150	1.8	1.6
01.03.05	9695	230	150	2.2	1.4
02.03.05	9908	190	190	1.8	1.9
03.03.05	9708	210	220	2.1	2.2
04.03.05	9062	250	240	2.2	2.2
05.03.05	8713	180	240	1.6	2.1
06.03.05	8118	250	270	2.0	2.2
07.03.05	13332	230	350	3.0	4.7
08.03.05	17474	160	270	2.7	4.7
09.03.05	17943	120	170	2.2	3.0
10.03.05	14675	120	140	1.8	2.0
11.03.05	23874	80	120	1.9	2.9
12.03.05	31214	60	66	1.9	2.1
13.03.05	31413	61	58	1.9	1.8
14.03.05	23829	43	58	1.0	1.4
15.03.05	18610	94	82	1.7	1.5
16.03.05	18880	52	90	1.0	1.7
17.03.05	21269	35	59	0.7	1.3
18.03.05	23865	36	49	0.9	1.2
19.03.05	24391	25	52	0.6	1.3
20.03.05	20040	16	28	0.3	0.6
21.03.05	18890	30	24	0.6	0.5
22.03.05	16082	38	46	0.6	0.7
30.03.05	18225	62	60	1.1	1.1

Table XIII: Date of sampling, flow rate and influent and effluent concentration and load of β-estradiol 3-sulfate in STP 2.

STP 2 β-Estradiol 3-sulfate					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [mg/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	<LOD	29	92	311
01.03.05	9695	<LOD	22	82	217
02.03.05	9908	<LOD	16	84	156
03.03.05	9708	<LOD	36	83	346
04.03.05	9062	<LOD	26	77	235
05.03.05	8713	<LOD	32	74	283
06.03.05	8118	8.5-28*	31	227	253
07.03.05	13332	<LOD	52	113	689
08.03.05	17474	<LOD	24	149	414
09.03.05	17943	<LOD	29	153	515
10.03.05	14675	<LOD	34	125	504
11.03.05	23874	<LOD	39	203	936
12.03.05	31214	<LOD	23	265	714
13.03.05	31413	<LOD	<LOD	267	57
14.03.05	23829	<LOD	23	203	545
15.03.05	18610	<LOD	24	158	451
16.03.05	18880	<LOD	30	160	569
17.03.05	21269	<LOD	14	181	298
18.03.05	23865	8.5-28*	<LOD	668	43
19.03.05	24391	8.5-28*	23	683	565
20.03.05	20040	8.5-28*	24	561	487
21.03.05	18890	<LOD	12	161	221
22.03.05	16082	<LOD	26	137	421
30.03.05	18225	8.5-28*	10	510	187

Table XIV: Date of sampling, flow rate and influent and effluent concentration and load of estrone3-sulfate in STP 2.

STP 2 Estrone 3-sulfate					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	12	44	0.1	0.5
01.03.05	9695	11	41	0.1	0.4
02.03.05	9908	11	60	0.1	0.6
03.03.05	9708	15	68	0.1	0.7
04.03.05	9062	19	61	0.2	0.6
05.03.05	8713	13	62	0.1	0.5
06.03.05	8118	14	66	0.1	0.5
07.03.05	13332	9.3	73	0.1	1.0
08.03.05	17474	7.7	58	0.1	1.0
09.03.05	17943	8.3	47	0.1	0.9
10.03.05	14675	9.8	49	0.1	0.7
11.03.05	23874	5.8	53	0.1	1.3
12.03.05	31214	6.2	31	0.2	1.0
13.03.05	31413	4.1	23	0.1	0.7
14.03.05	23829	6.5	37	0.2	0.9
15.03.05	18610	5.5	45	0.1	0.8
16.03.05	18880	8.3	44	0.2	0.8
17.03.05	21269	10	47	0.2	1.0
18.03.05	23865	6.5	42	0.2	1.0
19.03.05	24391	19	51	0.5	1.2
20.03.05	20040	6.5	38	0.1	0.8
21.03.05	18890	11	33	0.2	0.6
22.03.05	16082	11	37	0.2	0.6
30.03.05	18225	18	17	0.3	0.3

Table XV: Date of sampling, flow rate and influent and effluent concentration and load of estriol in STP 2.

STP 2 Estriol					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	12-35*	4.5-15*	0.7	0.3
01.03.05	9695	84	51	1.4	0.5
02.03.05	9908	38	100	0.6	1.0
03.03.05	9708	46	83	0.8	0.8
04.03.05	9062	54	47	0.8	0.4
05.03.05	8713	62	98	0.9	0.9
06.03.05	8118	67	36	0.9	0.3
07.03.05	13332	38	210	0.9	2.7
08.03.05	17474	75	220	2.2	3.8
09.03.05	17943	76	140	2.4	2.4
10.03.05	14675	92	110	2.3	1.5
11.03.05	23874	210	290	8.6	6.9
12.03.05	31214	110	33	5.8	1.0
13.03.05	31413	120	100	6.2	3.1
14.03.05	23829	110	74	4.4	1.8
15.03.05	18610	110	82	3.5	1.5
16.03.05	18880	200	180	6.3	3.4
17.03.05	21269	220	230	8.0	5.0
18.03.05	23865	460	430	19	10
19.03.05	24391	450	520	19	13
20.03.05	20040	270	400	9.2	8.1
21.03.05	18890	170	160	5.5	3.0
22.03.05	16082	68	220	1.9	3.6
30.03.05	18225	160	120	4.9	2.2

Table XVI: Date of sampling, flow rate and influent and effluent concentration and load of 16α-hydroxyestrone in STP 2.

STP 2 16α-Hydroxyestrone					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	110	25	1.2	0.3
01.03.05	9695	89	15	0.9	0.1
02.03.05	9908	130	27	1.3	0.3
03.03.05	9708	130	16	1.3	0.2
04.03.05	9062	82	62	0.7	0.6
05.03.05	8713	120	18	1.1	0.2
06.03.05	8118	170	80	1.4	0.7
07.03.05	13332	88	80	1.2	1.1
08.03.05	17474	96	73	1.7	1.3
09.03.05	17943	80	59	1.4	1.1
10.03.05	14675	170	32	2.5	0.5
11.03.05	23874	69	2.4-8*	1.6	0.0
12.03.05	31214	48	20	1.5	0.6
13.03.05	31413	26	15	0.8	0.5
14.03.05	23829	57	25	1.4	0.6
15.03.05	18610	86	<LOD	1.6	0.6
16.03.05	18880	37	2.4-8*	0.7	0.0
17.03.05	21269	33	2.4-8*	0.7	0.0
18.03.05	23865	33	2.4-8*	0.8	0.0
19.03.05	24391	2.4-8*	30	0.3	0.7
20.03.05	20040	39	2.4-8*	0.8	0.0
21.03.05	18890	30	2.4-8*	0.6	0.0
22.03.05	16082	53	21	0.9	0.3
30.03.05	18225	22	2.4-8*	0.4	0.0

Table XVII: Date of sampling, flow rate and influent and effluent concentration and load of 17β-estradiol in STP 2.

STP 2 17β-Estradiol					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [mg/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	2.4-8*	2.4-8*	86	86
01.03.05	9695	2.4-8*	12	78	112
02.03.05	9908	2.4-8*	15	79	152
03.03.05	9708	2.4-8*	2.4-8*	78	78
04.03.05	9062	<LOD	13	22	117
05.03.05	8713	2.4-8*	17	70	151
06.03.05	8118	2.4-8*	25	65	200
07.03.05	13332	2.4-8*	2.4-8*	107	107
08.03.05	17474	2.4-8*	14	140	140
09.03.05	17943	13	35	239	636
10.03.05	14675	13	11	188	161
11.03.05	23874	9.0	25	215	588
12.03.05	31214	2.4-8*	12	250	387
13.03.05	31413	2.4-8*	14	251	425
14.03.05	23829	8.1	18	194	438
15.03.05	18610	18	23	326	432
16.03.05	18880	10	32	197	609
17.03.05	21269	17	2.4-8*	356	170
18.03.05	23865	13	10	321	249
19.03.05	24391	9.4	14	228	346
20.03.05	20040	11	20	224	410
21.03.05	18890	11	18	200	335
22.03.05	16082	17	20	278	319
30.03.05	18225	17	13	318	244

Table XVIII: Date of sampling, flow rate and influent and effluent concentration and load of estrone in STP 2.

STP 2 Estrone					
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**		**Load [g/d]**	
		Influent	**Effluent**	**Influent**	**Effluent**
28.02.05	10795	33	67	0.4	0.7
01.03.05	9695	68	95	0.7	0.9
02.03.05	9908	49	101	0.5	1.0
03.03.05	9708	52	79	0.5	0.8
04.03.05	9062	68	65	0.6	0.6
05.03.05	8713	60	89	0.5	0.8
06.03.05	8118	43	174	0.4	1.4
07.03.05	13332	45	49	0.6	0.7
08.03.05	17474	52	96	0.9	1.7
09.03.05	17943	59	114	1.0	2.1
10.03.05	14675	80	99	1.2	1.4
11.03.05	23874	47	157	1.1	3.8
12.03.05	31214	45	88	1.4	2.7
13.03.05	31413	18	73	0.6	2.3
14.03.05	23829	38	105	0.9	2.5
15.03.05	18610	102	99	1.9	1.8
16.03.05	18880	69	129	1.3	2.4
17.03.05	21269	53	36	1.1	0.8
18.03.05	23865	37	60	0.9	1.4
19.03.05	24391	55	93	1.4	2.3
20.03.05	20040	61	90	1.2	1.8
21.03.05	18890	49	77	0.9	1.5
22.03.05	16082	71	79	1.1	1.3
30.03.05	18225	53	70	1.0	1.3

Table XIX: Date of sampling, flow rate and influent and effluent concentration and load of erythromycin in STP 3.

STP 3 Erythromycin							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	160	150	160	1.5	1.4	1.5
07.06.05	12117	160		130	1.9		1.6
08.06.05	10089	450		150	4.6		1.5
09.06.05	10079	330	160	130	3.3	1.6	1.3
10.06.05	11845	260		210	3.0		2.5
11.06.05	10749	220		260	2.3		2.8
12.06.05	9219	780		230	7.2		2.1
13.06.05	10778	240	200	240	2.5	2.1	2.6
14.06.05	9188	240		260	2.2		2.3
15.06.05	8865	300		250	2.7		2.2
16.06.05	6206	370	340	290	2.3	2.1	1.8
17.06.05	9364	290		330	2.8		3.0
18.06.05	8453	360		260	3.1		2.2
19.06.05	7944	530		190	4.2		1.5
20.06.05	8577	430	410	460	3.6	3.5	4.0
21.06.05	8633	340		360	2.9		3.1
22.06.05	8403	600		830	5.0		7.0
23.06.05	8398	350	320	330	2.9	2.7	2.7
24.06.05	10980	230		280	2.5		3.1
25.06.05	8750	400		370	3.5		3.3
26.06.05	7748	450		520	3.5		4.0
27.06.05	8525	430	310	310	3.7	2.6	2.7
28.06.05	7937	190		330	1.5		2.6
29.06.05	16601	220		180	3.6		3.0
30.06.05	33529	160	97	85	5.3	3.2	2.8
01.07.05	33782	85		80	2.9		2.7
02.07.05	23242	170		200	4.0		4.5
03.07.05	13651	210	150	240	2.9	1.4	3.2

Table XX: Date of sampling, flow rate and influent and effluent concentration and load of clarithromycin in STP 3.

STP 3 Clarithromycin							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	240	240	250	2.2	2.2	2.3
07.06.05	12117	320		210	3.9		2.5
08.06.05	10089	290		270	2.9		2.8
09.06.05	10079	280	260	260	2.8	2.6	2.6
10.06.05	11845	360		360	4.2		4.3
11.06.05	10749	480		350	5.1		3.8
12.06.05	9219	1500		300	14		2.8
13.06.05	10778	430	390	470	4.7	4.2	5.0
14.06.05	9188	300		470	2.8		4.3
15.06.05	8865	430		480	3.8		4.3
16.06.05	6206	520	440	480	3.2	2.7	3.0
17.06.05	9364	490		510	4.6		4.8
18.06.05	8453	390		480	3.3		4.1
19.06.05	7944	220		520	1.8		4.1
20.06.05	8577	240	350	450	2.1	3.0	3.8
21.06.05	8633	240		500	2.0		4.3
22.06.05	8403	230		440	1.9		3.7
23.06.05	8398	130	300	390	1.1	2.5	3.2
24.06.05	10980	210		360	2.3		4.0
25.06.05	8750	160		340	1.4		3.0
26.06.05	7748	160		360	1.2		2.8
27.06.05	8525	190	230	330	1.6	1.9	2.8
28.06.05	7937	93		310	0.7		2.5
29.06.05	16601	98		290	1.6		4.7
30.06.05	33529	92	89	190	3.1	3.0	6.5
01.07.05	33782	150		160	5.1		5.3
02.07.05	23242	370		160	8.7		3.6
03.07.05	13651	350	240	190	4.8		2.6

Table XXI: Date of sampling, flow rate and influent and effluent concentration and load of roxithromycin in STP 3.

STP 3 Roxithromycin							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	92	93	110	0.9	0.9	1.0
07.06.05	12117	81		87	1.0		1.1
08.06.05	10089	56		110	0.6		1.1
09.06.05	10079	55	97	91	0.6	1.0	0.9
10.06.05	11845	93		130	1.1		1.5
11.06.05	10749	78		120	0.8		1.3
12.06.05	9219	56		110	0.5		1.0
13.06.05	10778	72	149	130	0.8	1.6	1.4
14.06.05	9188	46		180	0.4		1.7
15.06.05	8865	66		150	0.6		1.3
16.06.05	6206	49	102	140	0.3	0.6	0.8
17.06.05	9364	71		120	0.7		1.1
18.06.05	8453	57		98	0.5		0.8
19.06.05	7944	65		97	0.5		0.8
20.06.05	8577	89	87	96	0.8	0.7	0.8
21.06.05	8633	72		100	0.6		0.9
22.06.05	8403	94		110	0.8		0.9
23.06.05	8398	50	97	120	0.4	0.8	1.0
24.06.05	10980	78		130	0.9		1.4
25.06.05	8750	49		130	0.4		1.1
26.06.05	7748	70		160	0.5		1.2
27.06.05	8525	72	110	170	0.6	0.9	1.5
28.06.05	7937	54		150	0.4		1.2
29.06.05	16601	50		150	0.8		2.5
30.06.05	33529	51	46	94	1.7	1.5	3.2
01.07.05	33782	59		56	2.0		1.9
02.07.05	23242	25		58	0.6		1.3
03.07.05	13651	84	93	85	1.1	0.9	1.2

Table XXII: Date of sampling, flow rate and influent and effluent concentration and load of β-estradiol 3-sulfate in STP 3.

STP 3 β-Estradiol 3-sulfate							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	<LOD	5.8	14	0.1	0.1	0.1
07.06.05	12117	<LOD		9.0	0.1		0.1
08.06.05	10089	8.5-28*		14	0.3		0.1
09.06.05	10079	8.5-28*	8.0	139	0.3	0.1	1.4
10.06.05	11845	8.5-28*		90	0.3		1.1
11.06.05	10749	<LOD		16	0.1		0.2
12.06.05	9219	8.5-28*		13	0.3		0.1
13.06.05	10778	8.5-28*	<LOD	141	0.3	0.0	1.5
14.06.05	9188	8.5-28*		9.9	0.3		0.1
15.06.05	8865	32		29	0.3		0.3
16.06.05	6206	8.5-28*	8.8	10	0.2	0.1	0.1
17.06.05	9364	79		14	0.7		0.1
18.06.05	8453	8.5-28*		12	0.2		0.1
19.06.05	7944	<LOD		23	0.1		0.2
20.06.05	8577	8.5-28*	6.8	5.6	0.2	0.1	0.0
21.06.05	8633	33		13	0.3		0.1
22.06.05	8403	8.5-28*		<LOD	0.2		0.0
23.06.05	8398	8.5-28*	7.7	13	0.2	0.1	0.1
24.06.05	10980	<LOD		5.2	0.1		0.1
25.06.05	8750	8.5-28*		13	0.2		0.1
26.06.05	7748	8.5-28*		14	0.2		0.1
27.06.05	8525	8.5-28*	0.6-1.8*	6.8	0.2	0.0	0.1
28.06.05	7937	<LOD		8.6	0.1		0.1
29.06.05	16601	<LOD		15	0.1		0.2
30.06.05	33529	8.5-28*	15.9	10	0.9	0.5	0.3
01.07.05	33782	30		17	1.0		0.6
02.07.05	23242	36		<LOD	0.8		0.0
03.07.05	13651	25		13	0.3		0.2

Table XXIII: Date of sampling, flow rate and influent and effluent concentration and load of estrone 3-sulfate in STP 3.

STP 3 Estrone 3-sulfate							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [mg/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	6.1	1.2	2.1	57	12	20
07.06.05	12117	5.5		2.1	67		25
08.06.05	10089	10		0.9	106		9
09.06.05	10079	14	0.8	2.0	142	8	20
10.06.05	11845	13		3.5	160		41
11.06.05	10749	7.4		2.0	79		22
12.06.05	9219	8.1		0.8	74		7
13.06.05	10778	4.6	5.0	<LOD	50	54	5
14.06.05	9188	12		2.9	111		27
15.06.05	8865	9.3		9.3	82		82
16.06.05	6206	15	3.0	3.0	93	19	18
17.06.05	9364	26		3.4	242		32
18.06.05	8453	8.6		4.6	73		39
19.06.05	7944	6.9		6.2	55		49
20.06.05	8577	9.8	2.6	3.7	84	22	32
21.06.05	8633	16		4.9	137		43
22.06.05	8403	14		4.3	116		36
23.06.05	8398	16	<LOD	2.8	138	0	23
24.06.05	10980	20		2.4	217		26
25.06.05	8750	11		2.2	99		19
26.06.05	7748	15		3.1	119		24
27.06.05	8525	17	1.0	1.5	144	9	13
28.06.05	7937	13		0.8	107		6
29.06.05	16601	1.2-4*		5.8	66		97
30.06.05	33529	<LOD	<LOD	<LOD	40	0	17
01.07.05	33782	<LOD		<LOD	41	12	17
02.07.05	23242	6.9		<LOD	160		12
03.07.05	13651	<LOD	1.2	1.7	16		23

Table XXIV: Date of sampling, flow rate and influent and effluent concentration and load of estriol in STP 3.

STP 3 Estriol							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	61	4.5-15*	4.5-15*	0.6	0.1	0.1
07.06.05	12117	12-35*		<LOD	0.4		0.1
08.06.05	10089	161		<LOD	1.6		0.0
09.06.05	10079	150	<LOD	<LOD	1.6	0.0	0.0
10.06.05	11845	94		32	1.1		0.4
11.06.05	10749	170		<LOD	1.9		0.0
12.06.05	9219	180		<LOD	1.7		0.0
13.06.05	10778	12-35*	120	251	0.4	1.3	2.7
14.06.05	9188	76		<LOD	0.7		0.0
15.06.05	8865	70		<LOD	0.6		0.0
16.06.05	6206	210	<LOD	<LOD	1.3	0.0	0.0
17.06.05	9364	440		4.5-15*	4.2		0.1
18.06.05	8453	140		4.5-15*	1.1		0.1
19.06.05	7944	12-35*		4.5-15*	0.3		0.1
20.06.05	8577	12-35*	<LOD	<LOD	0.3	0.0	0.0
21.06.05	8633	93		53	0.8		0.5
22.06.05	8403	12-35*		<LOD	0.3		0.0
23.06.05	8398	<LOD	86	4.5-15*	0.1	0.7	0.1
24.06.05	10980	<LOD		<LOD	0.1		0.0
25.06.05	8750	95		4.5-15*	0.8		0.1
26.06.05	7748	12-35*		29	0.3		0.2
27.06.05	8525	93	<LOD	4.5-15*	0.8	0.0	0.1
28.06.05	7937	<LOD		62	0.1		0.5
29.06.05	16601	12-35*		<LOD	0.6		0.1
30.06.05	33529	12-35*	46	4.5-15*	1.2	1.5	0.5
01.07.05	33782	100		104	3.5		3.5
02.07.05	23242	12-35*		<LOD	0.8		0.1
03.07.05	13651	390	4.5-15*	4.5-15*	5.3	0.1	0.2

Table XXV: Date of sampling, flow rate and influent and effluent concentration and load of 16α-hydroxyestrone in STP 3.

STP 3 16α-Hydroxyestrone							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	53	2.4-8*	2.4-8*	0.5	0.0	0.1
07.06.05	12117	31		2.4-8*	0.4		0.1
08.06.05	10089	53		2.4-8*	0.5		0.1
09.06.05	10079	50	2.4-8*	<LOD	0.5	0.1	0.0
10.06.05	11845	62		2.4-8*	0.7		0.1
11.06.05	10749	46		2.4-8*	0.5		0.1
12.06.05	9219	38		2.4-8*	0.3		0.1
13.06.05	10778	33	2.4-8*	2.4-8*	0.4	0.1	0.1
14.06.05	9188	53		2.4-8*	0.5		0.1
15.06.05	8865	55		2.4-8*	0.5		0.1
16.06.05	6206	37	2.4-8*	2.4-8*	0.2	0.0	0.0
17.06.05	9364	42		2.4-8*	0.4		0.1
18.06.05	8453	100		14	0.9		0.1
19.06.05	7944	79		2.4-8*	0.6		0.1
20.06.05	8577	190	2.4-8*	11	1.6	0.1	0.1
21.06.05	8633	56		2.4-8*	0.5		0.1
22.06.05	8403	68		2.4-8*	0.6		0.1
23.06.05	8398	81	2.4-8*	14	0.7	0.1	0.1
24.06.05	10980	89		2.4-8*	1.0		0.1
25.06.05	8750	22		2.4-8*	0.2		0.1
26.06.05	7748	62		2.4-8*	0.5		0.1
27.06.05	8525	110	2.4-8*	2.4-8*	0.9	0.1	0.1
28.06.05	7937	62		2.4-8*	0.5		0.1
29.06.05	16601	63		17	1.1		0.3
30.06.05	33529	15	12	2.4-8*	0.5	0.4	0.3
01.07.05	33782	17		14	0.6		0.5
02.07.05	23242	39		2.4-8*	0.9		0.2
03.07.05	13651	49		2.4-8*	0.7		0.1

Table XXVI: Date of sampling, flow rate and influent and effluent concentration and load of 17β-estradiol in STP 3.

STP 3 17β-Estradiol							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	2.4-8*	<LOD	2.4-8*	0.1	0.0	0.1
07.06.05	12117	<LOD		2.4-8*	0.0		0.1
08.06.05	10089	8.5		<LOD	0.1		0.0
09.06.05	10079	9.6	<LOD	<LOD	0.1	0.0	0.0
10.06.05	11845	11		<LOD	0.1		0.0
11.06.05	10749	2.4-8*		<LOD	0.1		0.0
12.06.05	9219	2.4-8*		2.4-8*	0.1		0.1
13.06.05	10778	15	2.4-8*	2.4-8*	0.2	0.1	0.1
14.06.05	9188	10		2.4-8*	0.1		0.1
15.06.05	8865	2.4-8*		2.4-8*	0.1		0.1
16.06.05	6206	9.5	2.4-8*	2.4-8*	0.1	0.0	0.0
17.06.05	9364	14		<LOD	0.1		0.0
18.06.05	8453	10		7.4	0.1		0.1
19.06.05	7944	15		2.4-8*	0.1		0.1
20.06.05	8577	18	2.4-8*	2.4-8*	0.2	0.1	0.1
21.06.05	8633	12		2.4-8*	0.1		0.1
22.06.05	8403	15		2.4-8*	0.1		0.1
23.06.05	8398	2.4-8*	2.4-8*	2.4-8*	0.1	0.1	0.1
24.06.05	10980	2.4-8*		2.4-8*	0.1		0.1
25.06.05	8750	9.5		2.4-8*	0.1		0.1
26.06.05	7748	15		2.4-8*	0.1		0.1
27.06.05	8525	12	2.4-8*	2.4-8*	0.1	0.1	0.1
28.06.05	7937	2.4-8*		2.4-8*	0.1		0.1
29.06.05	16601	2.4-8*		2.4-8*	0.1		0.1
30.06.05	33529	2.4-8*	2.4-8*	2.4-8*	0.3	0.3	0.3
01.07.05	33782	2.4-8*		2.4-8*	0.3		0.3
02.07.05	23242	2.4-8*		2.4-8*	0.2		0.2
03.07.05	13651	10		2.4-8*	0.1	0.0	0.1

Table XXVII: Date of sampling, flow rate and influent and effluent concentration and load of estrone in STP 3.

STP 3 Estrone							
Date	**Flow-rate [m³/d]**	**Concentration [ng/L]**			**Load [g/d]**		
		Influent	**Sett.-tank**	**Effluent**	**Influent**	**Sett.-tank**	**Effluent**
06.06.05	9313	46	21	24	0.4	0.2	0.2
07.06.05	12117	43		18	0.5		0.2
08.06.05	10089	61		20	0.6		0.2
09.06.05	10079	60	24	15	0.6	0.2	0.1
10.06.05	11845	70		22	0.8		0.3
11.06.05	10749	63		24	0.7		0.3
12.06.05	9219	40		28	0.4		0.3
13.06.05	10778	74	34	29	0.8	0.4	0.3
14.06.05	9188	54		29	0.5		0.3
15.06.05	8865	62		62	0.5		0.5
16.06.05	6206	78	37	30	0.5	0.2	0.2
17.06.05	9364	87		32	0.8		0.3
18.06.05	8453	45		36	0.4		0.3
19.06.05	7944	50		27	0.4		0.2
20.06.05	8577	68	41	37	0.6	0.4	0.3
21.06.05	8633	45		34	0.4		0.3
22.06.05	8403	53		32	0.4		0.3
23.06.05	8398	43	38	27	0.4	0.3	0.2
24.06.05	10980	55		29	0.6		0.3
25.06.05	8750	67		23	0.6		0.2
26.06.05	7748	54		26	0.4		0.2
27.06.05	8525	66	29	26	0.6	0.2	0.2
28.06.05	7937	35		22	0.3		0.2
29.06.05	16601	31		24	0.5		0.4
30.06.05	33529	14	17	12	0.5	0.6	0.4
01.07.05	33782	22		18	0.7		0.6
02.07.05	23242	45		16	1.0		0.4
03.07.05	13651	43		12	0.6		0.2