

Läkemedelsrester i två reningsverk och i recipienten Viskan



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Förord

Dessa två rapporter är resultatet av ett 20 p examensarbete vid Umeå universitet samt ett fortsatt projekt av samma student. I den första rapporten studeras antibiotikarester och i den andra rapporten hormoner. Det finns naturligtvis många andra ämnen att studera, men vi har i denna förstudie inriktat oss på ett urval av antibiotika och östrogena ämnen. Det är dock intressant att konstatera att laboratoriet som analyserade östrogenerna också fann tydliga toppar av blodtryckssänkande mediciner och värkhämmande medel (paracetamol) samt otydliga toppar av lugnande medel i Viskans vatten i utloppet vid Åsbro.

Rapporten kommer att användas i Marks kommuns och Borås Stads arbete för en förbättrad vattenkvalitet i Viskan. Författaren är ensam ansvarig för innehållet i rapporten.

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**Analyser av ett urval antibiotika i
Gässlösa reningsverk och Skene reningsverk
samt i recipienten Viskan**

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Umeå universitet 2005

Sammanfattning

Förekomsten av fem olika antibiotika för humant bruk har undersökts i två reningsverk i Marks och Borås kommun, samt i recipienten Viskan. Veckoprover av både orenat och renat avloppsvatten samt vatten och sediment från recipienten har analyserats. De antibiotika som undersökts är tre av fluorokinolontyp – ciprofloxacin, norfloxacin och ofloxacin – samt trimetoprim och sulfametoxazol. För analys av ämnena har vätskekromatografi i kombination med tandem-masspektrometri använts. Massflöden av ämnena i reningsverken har beräknats, dessutom har konsumtionsdata för de olika antimikrobiella substanserna använts för att kunna jämföra PEC – de förväntade koncentrationen i miljön – med det uppmätta värdet (MEC). PEC har också använts för att kunna genomföra en preliminär riskbedömning av föreningarna.

Spridningen av läkemedel till miljön kan ske genom ett flertal olika vägar. Vanligast är dock att vi människor metaboliserar läkemedlet till olika grad i kroppen och att dessa restprodukter, vilka till stor del kan bestå av oförändrade, aktiva substanser, följer med urin och avföring ut i avloppsvattnet. Reningsverken klarar inte av att rena bort alla dessa ämnen, och läkemedlen kan då följa med ut i recipienten, där de kan utöva olika negativa effekter på de organismer som lever där.

Antibiotika verkar genom olika mekanismer som inhiberar eller dödar bakterier. De kan ha en lång livslängd i miljön, beroende på vilka egenskaper de har. Det finns många exempel på förekomst av antibiotika i miljön och i reningsverk. De har påträffats i slam från reningsverk, i jord, vattendrag, sediment och även i små mängder i fisk som lever nära utloppen från reningsverk. De flesta substanser som påträffats i miljön tros ha sitt ursprung i avloppsvatten, men även spridning av avloppsslam och gödsel från antibiotikabehandlat boskap leder till avsättning av dessa substanser i naturen. En av de negativa effekterna kopplade till antibiotikaanvändning är utvecklingen av resistens, som förefaller öka med ökad användning. Förekomsten av resistens i naturen tros ha sitt ursprung i spridningen av resistent bakterier från reningsverk. Klara bevis för att reningsverk gynnar utvecklingen av resistens saknas dock. Antibiotika tros emellertid kunna påverka reningsprocesserna i avloppsverken på deras antimikrobiella effekt.

Negativa effekter framkallade av antibiotika drabbar inte bara bakterier, även andra arter har visats sig kunna angripas. Organismer långt ner i näringskedjorna, t ex hinnkräftor av släktet *Daphnia* samt olika algar, uppvisar toxiska effekter vid låga antibiotikakoncentrationer. Även organismer som uppehåller sig i jorden drabbas av substanserna negativa effekter. Påverkan på organismer långt ner i näringskedjor förmodas kunna beröra hela ekosystem.

Analysen av vattenproverna visade att trimetoprim förekom i lika hög koncentration både i in- och utvattnet från reningsverken – mellan 112 och 166 ng/L, medan förekomsten av fluorokinoloner inte har kunnat påvisas i det renade vattnet. Ingående koncentrationer i verken varierade mellan icke detekterbart för ofloxacin i Gässlösa och 473 ng/L för ciprofloxacin i Skene. Över lag var fluorokinolon-koncentrationerna högre i Skene

reningsverk än i Gässlösa, vilket kan tyda på en högre användning av dessa substanser i det området. Massflödena visade också på att högre mängder av de antibiotiska substanserna konsumeras i området för Skene reningsverk. Sulfametoxazol kunde inte påvisas i något av avloppsproverna.

Inget av vattenproverna från Viskan uppvisade detekterbara halter av någon av de undersökta substanserna. Dock har analysen av dricksvatten från Borås, som användes som blank-prov, avslöjat koncentrationer över kvantifieringsnivån för tre av de undersökta kinolon-föreningarna. Vad som är orsaken till detta resultat är svårt att säga, men tros inte enbart bero på kontamination av proverna under laborationsarbetet. Resultaten från sedimentproverna kunde inte användas för att beräkna koncentrationer. Provmatrisen förmodas ha reagerat med kolonnmaterialiet, vilket gav en stor fördröjning av retentionstiderna. Resultatet från första provkörningen, vilket inte uppvisade lika fördröjda retentionstider, visade dock att kvoten mellan areorna för analyt och intern standard är så liten att koncentrationerna kan antas vara under kvantifieringsnivåerna för respektive substans. Den metod som använts för att upparbeta sedimentproverna förutsätts inte vara lämplig, varför en alternativ metod bör nyttjas för att kunna analysera föreningar i denna matris.

En jämförelse mellan PEC och MEC visade att den förväntade koncentrationen starkt överstiger den uppmätta koncentrationen. En förfinad variant av PEC ger en mer realistisk bild och en koncentration närmare MEC. Beräkning av den förväntade koncentrationen av de två icke analytiskt undersökta fluorokinolonerna som används inom svensk sjukvård – levofloxacin och moxifloxacin – uppvisade PEC under kvantifieringsnivå.

Resultaten från riskbedömningen visade att inget av de analyserade ämnena kan förväntas utgöra någon risk för miljön, varken när de förekommer i de beräknade, förväntade nivåerna eller i de uppmätta halterna. Riskbedömningar och ekotoxicitetstest ska dock ses som ett simplificerat verktyg för att avgöra ett ämnes farlighet, eftersom de aldrig kan svara för alla de reaktioner och effekter ett ämne genomgår och utövar i naturen.

Abstract

Five antibiotic substances for human use, including the fluoroquinolones (FQs) ciprofloxacin, norfloxacin and ofloxacin together with trimethoprim and sulfamethoxazole, were analysed in two sewage treatment plants (STPs) and in the recipient, the small river of Viskan. Weekly samples of both raw sewage and final effluent from the STPs, as well as water and sediment from the recipient were analysed. The measured concentrations were compared with consumption and effect data obtained in the study. A preliminary risk assessment was thereafter implemented.

Antibiotic substances can be detected in several different environmental compartments, e.g. surface water, soil and sediment. The antibiotics are supposed to reach the environment primarily through discharge of sewage water, when sewage sludge is used as a soil improving agent or with manure from animals treated with antibiotics. A relation between the presence of antibiotics and the emergence of antibiotic resistance has been found. Many of the bacterial strains present in STPs show resistance towards one or more antibiotics. These substances are also believed to disturb processes in STPs due to their antimicrobial effect. Furthermore, antibiotic substances have shown negative effects on non-target organisms. Various primary producers appear to be affected, which could have consequences for entire ecosystems.

The water and sediment samples were extracted using solid phase extraction and liquid/solid extraction respectively. The analyses were performed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Concentrations of the substances varied from 473 ng/L for ciprofloxacin to 6 ng/L for norfloxacin in the raw sewage. In the final effluent, only trimethoprim was detected. Trimethoprim is not reduced during the water cleaning process and concentrations were just above 100 ng/L in all sewage samples. Sulfamethoxazole were below its' limit of quantification in all samples and ofloxacin was only found in the raw sewage of one of the plants.

A comparison of the measured concentrations and predicted environmental concentrations (PECs) showed large differences in the values. A refined PEC will give a more accurate prognosis of the environmental concentrations. Calculating risk by using the EMEA guideline, revealed that none of the substances constitute a risk to environmental organisms at the concentrations calculated or measured.

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1. Introduction

More than one thousand tonnes of active pharmaceutical substances are used in Sweden every year [1]. During 2004, Apoteket AB, which has the exclusive rights to sell drugs in Sweden, sold almost 29 billion defined daily doses of pharmaceuticals [2]. Of these 29 billions, almost 1 billion doses were antimicrobial substances, used to prevent and treat infections caused by microbes in both human and animal medicine. The term antimicrobial, or more commonly “antibiotic”, describes a medicine which destroys or inhibits the growth of bacteria [3]. The amount of antibiotics used increases every year – the yearly consumption of active antibiotic substances reaches 70 tonnes in Sweden [4] and the worldwide antibiotic use is estimated to lie between 100 000 and 200 000 tonnes [5]. The most prescribed groups of antibiotics in Sweden are the fluoroquinolones, different types of penicillins and the tetracyclines, which are also used for animal treatment [2].

Using antibiotics for treating infectious diseases is a less than one hundred years old phenomenon [6]. The breaking point came with Fleming’s discovery of the antimicrobial effect of penicillin. Nowadays, the scope of antimicrobial substances for fighting infectious disease has increased to a number of more than 160 compounds. The negative effects from using antibiotics occurred as early as half a century ago, just a few years after penicillin was put on the market [7]. Scientists began to notice the emergence of a penicillin-resistant strain of *Staphylococcus aureus*, a common bacterium present in the human body’s normal bacterial flora. Since then, resistance has grown to become a serious problem for controlling infectious diseases.

After distribution, the main fate of pharmaceuticals is to pass the body either as metabolites or in unchanged, active form, and thereafter end up in the municipal sewage treatment plants (STPs) [3, 8]. The modern treatment plants are designed to prevent eutrophication and to avoid the spreading of contagious diseases [9]. The wastewater is therefore purified from oxygen demanding substances, nitrogen and phosphorous containing compounds and microbes. The cleaning processes utilized today are not sufficient when concerning other substances than the naturally occurring organic compounds [3]. A large quantity of xenobiotics, i.e. substances with non-natural origin, including antibiotics, reaches the environment after passing through the STP into the receiving water, where they can exert various negative effects. This certainly concerns pharmaceuticals, whose main purpose is to provoke biological effects. Antibiotic substances and their residues are not only found in the water and sludge in sewage plants [8, 10] – they have also been detected in surface water [3, 10] ground water [10] and drinking water [3]. However, data on the behaviour and the fate of pharmaceuticals in rivers and lakes, including the sediment compartment, are extremely rare [11]. The measured concentrations of antibiotics in the environment fluctuate from milligram per litre [mg/L] in hospital sewage water, to a few nanograms per litre [ng/L] in surface and ground water [8].

During the last decade, problems concerning pharmaceutical residues in the environment have attracted attention, and research within this area is increasing [1]. The antibiotic substances have caused special concern due to their negative influence on resistance development in bacteria. The Swedish National Board of Health and Welfare state that, in comparison to the development of bacterial resistance, other health issues associated with pharmaceuticals in the environment are negligible. The effect of antibiotics on the emergence of resistance is evident and toxicity of antibiotics against pathogenic bacteria is well known [12]. However, little data exist about the adverse effects the substances cause to the wastewater treatment process, the microbial life in surface waters and their toxicity at low concentrations to non-target organisms.

The European Medicines Agency (EMA) published in 2001 a draft guideline for implementing environmental risk assessments (ERAs) of medicinal products [13-15]. The draft describes a stepwise procedure for the assessment, including calculation of the predicted environmental concentration (PEC) and analysis of environmental fate and effects by estimating the predicted no effect concentration (PNEC) [14]. Risk assessments for antibiotics and other pharmaceuticals have gradually emerged in literature during this last decade [16, 17]. In 2004, the Swedish Medical Product Agency released a report [18] on environmental effects from pharmaceuticals and personal care products, stating that there is not enough knowledge concerning pharmaceuticals in the environment. There is, according to this report, a lack of information about long-term ecotoxic effects and the presence of pharmaceuticals in the environment. Also, the models for predicting concentrations and effects must be improved to be useful.

1.1. Aim

The object of this project was to:

- 1) determine concentrations and mass flows of three fluoroquinolones, sulfamethoxazole and trimethoprim in effluents from two sewage treatment plants (Skene and Gässlösa) and in the recipient (Viskan) waters and sediments
- 2) use regional consumption data of all fluoroquinolones and sulphonamides to calculate the predicted environmental concentrations (PEC)
- 3) compare measured concentrations with consumption and effect data obtained in the study to implement a preliminary risk assessment.

2. Background

2.1. Antibiotics

The word antibiotic is generally used for substances which act by inhibiting the growth of (bacteriostatic antibiotic) or killing (bactericide antibiotic) bacteria [19]. Since the substances do not affect mammalian cells, they can be used as medicinal products for bacterial infections in humans and livestock animals. Many antibiotics have originally been extracted from naturally occurring microorganisms, such as fungi and bacteria – for

example the penicillins. Other antibiotic substances are entirely synthetically produced, e.g. the fluoroquinolones. Most of the 160 different antibiotics used today are variations of 16 fundamental substances, which all use one of five common mechanisms for inhibition or killing of bacteria [19, 20]. Penicillins and vancomycin work through the most employed mechanism of action – inhibiting the synthesizing of the cell wall, which will lead to death of the bacterium. Tetracyclines are examples of a substance group which will enter a bacterium and attach to the ribosomes, thereby interfering with the organisms' ability to form proteins. When the protein synthesis is blocked, the bacteria cannot grow nor reproduce. Other antibiotics enter the cell, bind to and change the bacterial DNA. Antibiotics can also act by inhibiting enzymes involved in the bacterial metabolism, for example the sulfa substances. Another group of substances can change the permeability of the cell membrane, leading to a porous membrane that can leak of important substances.

Bacteria are divided into two major groups – gram-positive and -negative [19]. Gram-negative bacteria are characterized by an outer membrane and a thin peptidoglycan cell wall that stains poorly with a stain invented by a Danish physician. Gram-positive bacteria have no outer membrane and a thick peptidoglycan layer that stains well with the Gram stain. A broad spectrum antibiotic acts on both gram-negative and -positive bacteria. An antibiotic with a limited spectrum of activity can be useful for the control of microorganisms that fail to respond to other antibiotics.

2.1.1. The Fluoroquinolones

Since the introduction of the fluoroquinolones (FQs), the use of these antibiotics has rapidly increased and they are now some of the most used antimicrobials for many types of bacterial infections [21]. The FQs are a group of synthetically produced antibiotics commonly used in both human and veterinary medicine [22]. The FQs work through obstructing bacterial DNA-metabolism by inhibiting two key enzymes – Topoisomerase II, also known as DNA gyrase, and Topoisomerase IV. The topoisomerases solve the structural problems associated with DNA replication, transcription, recombination, and reparation [23]. Inhibiting the enzymes will eventually result in breakdown of the DNA, leading to irreversible damage and finally death of the bacterium.

The fluororquinolones are active against a wide range of gram-positive and -negative bacteria, comprising most clinically important pathogens, such as *Streptococcus pneumoniae*, *E. coli*, and *Chlamydia spp* [22]. Because of their broad spectrum, fluoroquinolones are commonly used to treat infections within the urinary tract and for treatment of pneumonia. The FQs are predominantly metabolized in the kidney and thereafter excreted via urine [24]. The substances are either metabolized to less active compounds, or will be excreted in their unchanged, active form. The degree of elimination and metabolism differs within the substance group (see Table 1).

There are five fluoroquinolones available on the Swedish market today – ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin and ofloxacin [2]. Ofloxacin is a racemate,

whereas levofloxacin is the pure enantiomere and the active component of ofloxacin. The total amount of FQ active compounds used can be seen in Table 1.

2.1.2. The Sulphonamides and Trimethoprim

Sulphonamides (SAs) are structural analogues of the para-aminobenzoic acid, PABA, which is an intermediate in the synthesis of folic acid in bacteria [22]. Because of their structural similarity, the sulfonamides block the conversion of PABA to the co-enzyme dihydrofolic acid, a reduced form of folic acid. The blocking of dihydrofolic acid decreases the amount of metabolically active tetrahydrofolic acid, a cofactor for the synthesis of purines, thymidine and DNA.

Susceptible bacteria are those who must synthesize folic acid. In mammals, dihydrofolic acid is obtained from dietary folic acid; thus sulfonamides do not affect human cells [24].

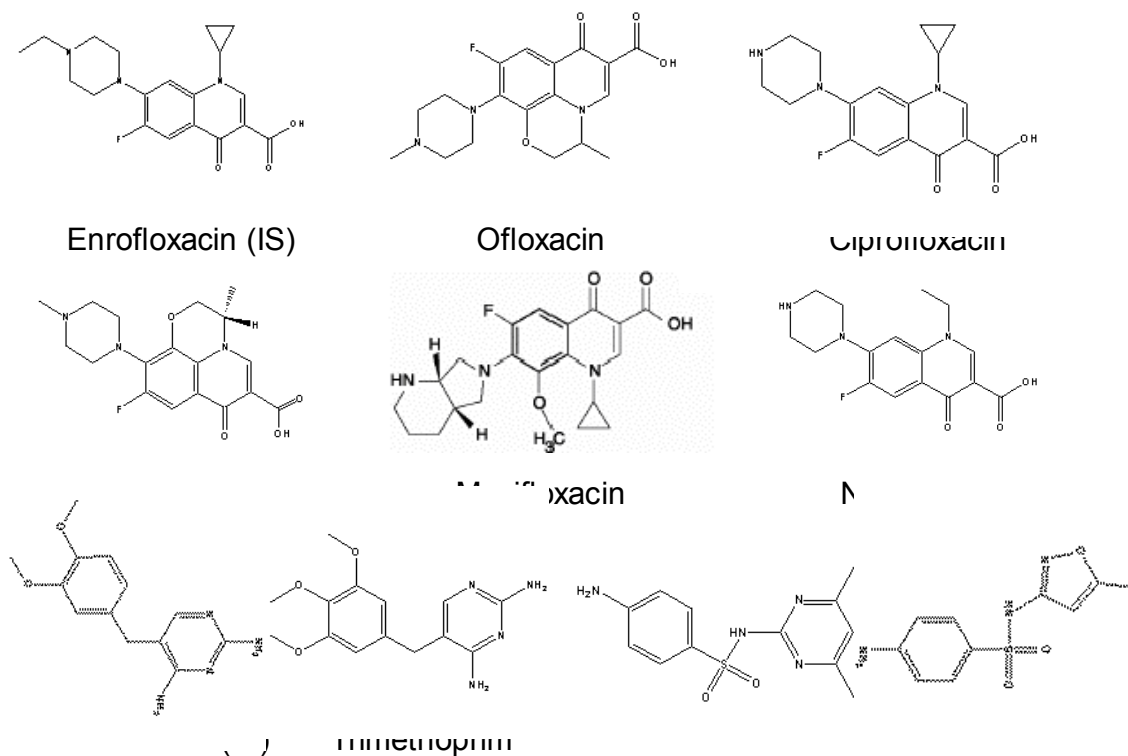


Figure 1. Chemical structure of the antibiotics and their respective internal standard (IS) [24, 25].

Trimethoprim binds to and reversibly inhibits the bacterial enzyme dihydrofolate reductase [22]. The inhibition leads to a blocking of the conversion of dihydrofolic acid to its functional form, tetrahydrofolic acid. The combination of sulphonamide/trimethoprim will block two consecutive steps in the folic acid metabolism, thus the synthesis of purine, RNA and DNA will be interrupted in microorganisms [24]. This form of sequence blockade will give a bactericide effect at concentrations where the two components individually would only exert biostatic effect.

The mechanism of action will complicate the development of resistance and the combination of substances is often more effective against organisms that are resistant against either of the components.

Trimethoprim and sulphonamides are effective against both gram-positive and -negative bacteria and are therefore used to treat urinary tract infections, chronic bronchitis and typhoid fever. Like the FQs, sulphonamides and trimethoprim are principally eliminated renally [22]. Small amounts of the compounds are excreted in bile and faeces.

The sulfonamides used in Sweden today are sulfamethoxazole and, to a negligible extent, sulfadiazine, which is only used in animal treatment [2]. Trimethoprim is very commonly used. The amount active compound used can be seen below in Table 1.

Table 1. *Excretion and consumption data on the antibiotics of interest [2, 22, 24].*

Substance	Antibiotic subgroup	Excretion of active compound	Mass active compound used in Sweden ^a [kg/year]
Ciprofloxacin	FQ	45%	3 200
Norfloxacin	FQ	30%	2 200
Ofloxacin	FQ	80%	80
Levofloxacin	FQ	85%	195
Moxifloxacin	FQ	20%	110
Trimethoprim	none	50%	2 900
Sulfamethoxazole	SA	30%	1 050

^aThe mass active compound used in Sweden is calculated by using the total number of defined daily doses (DDD) for pharmaceuticals used in human medicine, sold during 2004 in Sweden [2]. The number of DDD is then multiplied by the conversion factor (CF) of the drug, developed by the World Health Organisation, WHO. The CF used for “general anti-infectives for system use” is 1 500 mg/DDD [26].

2.2. The antibiotics’ physical and chemical properties

Generally, both abiotic and biotic processes determine the fate of the antibiotics in the aquatic environment [27]. Abiotic transformation in surface waters can occur via hydrolysis and photolysis, but since pharmaceuticals are designed for oral intake, they are usually resistant to hydrolysis, suggesting that photolysis is the primary pathway for their abiotic transformation in water. Photolysis is dependent on water turbidity, shading and depth, as well as seasonal changes in sunlight exposure [28]. Tests of the substances’ photodegradation reveal that both sulfamethoxazole and ofloxacin undergo fast degradation with half-lives ($t_{1/2}$) of 3 respectively 11 days [29]. However, photodegradation is of minor importance in sewage waters, which is not exposed to direct sunlight [30].

Besides chemical and photodegradation, biodegradability, i.e. decomposition by organisms, is an important aspect of the compounds’ persistence in the environment [31]. Biodegradation is based on the activity of microorganisms, which can be negatively

affected by the presence of antibiotics. 18 different antibiotics, including ofloxacin, sulfamethoxazole and trimethoprim, were tested for biodegradability – all substances were classified as not readily biodegradable, since they degraded by less than 60% during a test period of 28 days. Biodegradation by microorganisms is an important process for removing organic substances from sewage water in STPs [30]. However, laboratory experiments have shown that biodegradation of antibiotics in STPs might not be a reliable expectation for their removal.

Table 2. *Physical and chemical properties of the antibiotics and their internal standards (IS).*

Substance	CAS RN	Mw ^a	Water solubility		Log K _{ow} ^b	K _{d soil-water}	Stability in the environment
			[mol/g]	[mg/L]			t _{1/2} [days]
Enrofloxacin (IS)	93106-60-6	359,4	n.d.		1,1	260-6310	n.d.
Ciprofloxacin	85721-33-1	331,3		323	0,28	420	101-364
Norfloxacin	70458-96-7	319,3	178 000		-1	n.d.	101-364
Ofloxacin	83380-47-6	361,4	n.d.		0,35	309	101-364
Levofloxacin	100986-85-4	361,4	11 500		n.d.	n.d.	n.d.
Moxifloxacin	n.d. ^c	n.d.	n.d.		n.d.	n.d.	n.d.
Diaveridine (IS)	5355-16-8	260,3	3080		0,97	n.d.	n.d.
Trimethoprim	738-70-5	290,3	400		0,91	n.d.	20-100
Sulfamethazine (IS)	57-68-1	277,3	1500		0,89	1,3	n.d.
Sulfamethoxazole	723-46-6	253,3	610		0,89	0,22-1,8	>365
Reference	[25]	[25]	[25]		[17, 32]	[33]	[34]

^aMolecular weight

^bOctanol-Water Partition Coefficient. The octanol-water partition coefficient is the ratio of the concentration of a chemical in octanol and in water at equilibrium and at a specified temperature. This parameter is used in many environmental studies to help determine the fate of chemicals in the environment, since it imitates the biota lipid/water partition process [35].

^cNo data.

The persistence of a drug in sediment, sludge or soil is mostly dependent on its photostability, binding and adsorption capability and degradation rate [35]. The sorptive exchange of chemicals between a water phase and a solid phase is represented by the sorption coefficient, K_{d solid}, which is defined as the ratio between the concentration of the substance in the sorbent and in the water at equilibrium. A high K_{d solid} value corresponds to strong sorption to the solid phase. The values in Table 2 show that the FQs tend to bind stronger to the solid phase than do sulfamethoxazole. This has been confirmed by fate and mobility tests, where ofloxacin adsorbed the strongest to active sludge, whilst sulfamethoxazole adsorbed very little in all solid matrices studied [32]. Substances sorbed to sediments are no longer susceptible to photochemical degradation and experiments have also shown that FQs are very persistent in marine sediment compared to sulfonamides and trimethoprim [36].

2.3. Antibiotic use in agriculture

Antibiotics are used in animal husbandry for treating illness and disease, for prophylaxis purpose and growth promoting [37]. According to the European Commission Research Centre [38], 48% of the total consumption of antibiotics in the EU and Switzerland in the end of the 90's was used in animal farming. In the USA, also nearly fifty percent of the antibiotics consumed are employed in agriculture, mainly for animals, but also, to some content, for treating and preventing bacterial diseases in crops, fruit trees and ornament plants.

The Swedish Parliament has determined that antibiotics and chemotherapeutical substances can only be added to animal feed to prevent, indicate, mitigate or cure disease or symptoms of disease [39]. The Parliament also agreed to ban the addition of growth promoting substances to feed from the 1st of January 1986. In 1998, the European Union banned the antibiotics used for growth promoting assigned for human treatment, or that are known to select for cross-resistance to antibiotics which, for the time being, are used in human medicine [38]. According to the new additive decree, the use of growth promoting antibiotics must cease in the EU on the 31st of December 2005 [39]. In the USA, similar legislation has not been proposed and antibiotics used in human therapy, such as tetracyclines and penicillins, are also used as growth promoters [38]. In the EU, clinically important drugs, such as tetracyclines, penicillins and fluoroquinolones, are still used both for treating humans and animals. However, the fluoroquinolones analyzed in this project, are not used in stock farming in Sweden, and the detected concentrations can therefore only be a result from usage within human medicine [2]. Trimethoprim is, on the other hand, applied for treating infections in cattle, pig, sheep and poultry. Sulfamethoxazole is not used in animal medicine, but is replaced by another sulphonamide, sulfadiazine, which is currently not available for human medicine.

2.4. Occurrence of antibiotics in the environment

Table 3 demonstrates some of the measured concentrations of the target antibiotics in different aquatic environments. Their occurrence in surface water can foremost be derived from STP discharge, but also from farmland runoff, via manure and sewage sludge used as fertilizer [10]. Even groundwater, which is often used for drinking water, can be exposed to these pharmaceuticals through farmland leaching. Different pharmaceutical compounds have been detected in drinking water, which could indicate that antibiotics are also present in that compartment [3, 18]. Zuccato et al have found three antibiotics – erythromycin, spiramycin and tylosin – in Italian river sediments [40]. These drugs are used in animal farming, and the sampling was located in a densely populated area with many farms. It is therefore believed that the antibiotics in the sediment originate from animal feed.

Penicillin, chlortetracycline, streptomycin and tylosin are some of the antibiotics detected in the soil compartment [3, 41]. These substances are supposed to be due to the applying of manure and sewage sludge onto farmland. The fate of antibiotics in soil depends on the

compound – some bind to the soil, some are transported into streams with precipitation whilst others are degraded to metabolites. Antibiotics used in aquaculture will also lead to a discharge of the drugs in surface waters. Oxytetracycline, often used as a feed additive, has been detected in many investigations of sediments [3, 36, 42] so has the fluoroquinolone flumequine [3, 42], sometimes in concentrations up to several hundred micrograms per kilo ($\mu\text{g}/\text{kg}$) dry sediment weight. In Sweden low concentrations – 7,0-8,5 ng/g wet weight – of ciprofloxacin has been detected in fish which dwell in the waters close to a STP outflow [34].

Table 3. Concentrations of the target antibiotics in sewage water and in the environment, derived from literature.

Substance	Concentration [ng/L]	Compartment	Reference
Norfloxacin	66-155	raw sewage (Sweden)	[8]
	7-37	final effluent STP (Sweden)	[8, 43]
	250-553	raw sewage (Switzerland)	[44]
	51-73	final effluent STP (Switzerland)	[44]
	120	river (USA)	[45]
	3,3	inland sea (Sweden)	[43]
Ofloxacin	19-213	raw sewage (Sweden)	[8]
	7-52	final effluent STP (Sweden)	[8]
	2,1- 6,0	inland sea (Sweden)	[43]
Ciprofloxacin	90-300	raw sewage (Sweden)	[8]
	7-60	final effluent STP (Sweden)	[8]
	313-568	raw sewage (Switzerland)	[44]
	62-106	final effluent STP (Switzerland)	[44]
	14-26	river (Italy)	[46]
	200	river (USA)	[45]
Sulfamethoxazole	231-674	raw sewage (Sweden)	[8]
	135-304	final effluent STP (Sweden)	[8]
	200 (max value)	final effluent STP (Germany)	[10]
	470 (max value)	ground water (Germany)	[10]
	150	river (USA)	[45]
Trimethoprim	99-1300	raw sewage (Sweden)	[8]
	66-1340	final effluent STP (Sweden)	[8]
	660 (max value)	final effluent STP (Germany)	[10]
	200 (max value)	ground water (Germany)	[10]
	5,8-8,8	inland sea (Sweden)	[43]
	150	river (USA)	[45]

However, the compounds presented in Table 3 are not the only antibiotics found in the aquatic environment. The results presented by Calamari et al [46] also revealed detected

concentrations in the nanogram per litre range for antibiotics used in either human or animal treatment, e.g. erythromycin (human), lincomycin and oxytetracycline (animal). The sample points were located in the river Po, which surroundings are highly populated and there is a large number of animal farms situated close to the river. Kolpin et al [45] found 14 different antibiotics, e.g. five different sulfadruugs and tetracycline, in 139 different streams located in areas with intense urbanization and livestock production. A German investigation [47] of pharmaceuticals in groundwater revealed concentrations over 10 ng/L for several antibiotics, including several β -blockers and sulfonamides. The authors concluded that the compounds found in the groundwater are mainly due to the direct impact of wastewater. This conclusion was supported by the fact that the pattern of compounds found in the groundwater is the same as that found in many surface waters where the impact of wastewater is more evident. There are many other examples where antibiotic residues are found in environmental compartments [10, 28, 48].

2.5. Antibiotic resistance

Antimicrobial drug resistance is either an intrinsic or an acquired, natural occurring ability of an organism to resist the effects of a pharmaceutical to which it is normally susceptible [19, 38]. Since the antibiotics were introduced into medical practise, the prevalence of resistance in bacteria has increased worldwide. It is believed that resistance mechanisms have evolved from genes originally present in organisms producing antibiotics. The genes giving the ability to perform these mechanisms can, under the right circumstances, be transferred to neighbouring species which acquire those genes, or develop new mechanisms, to protect themselves from the inhibitory effects of the antibiotics to which they are exposed. It is now evident that horizontal gene transfer is coupled to the selective pressure caused by the presence of these substances in the environment [49]. The broad use of antibiotics have lead to a strong selective pressure, which has resulted in the survival and spreading of resistant bacteria [38, 50]. Until twenty years ago, resistance was concentrated in the hospitals due to the intensive use of antibiotics there, compared to the community. Today resistance has become widespread among community-acquired pathogens and commensal bacteria. During the 1970s, the emergence of organisms resistant not only to one type of antibiotics, but multiple other classes of antimicrobial agents, started to increase [38, 51]. The past years, a handful of organisms resistant to all known antibiotics have emerged. Infections caused by some strains of *Enterococci* and *Acinetobacter* are now virtually untreatable.

2.5.1. Resistance in the environment

Resistant bacteria and genetic material associated with resistance have been found in several different environmental compartments other than sewage effluents – for instance soil, sediment, surface and ground water [52]. A relation between the presence of antibiotics and the emergence of antibiotic resistance has been found. However, little is known about the effects of subinhibitory concentrations of these compounds on environmental bacteria, especially with respect to resistance. The bacteria already resistant, due to use of antibiotic in human and veterinary medicine, are considered a

more important source of resistant bacteria than the occurrence of these substances in nature. Resistance is nowadays a common problem within medical treatment, leading to increased clinical complications, prolonged stay in hospital, risk for serious diseases impossible to treat and a considerably increased cost for society [7, 53]. A possible scenario is that pathogenic bacteria will eventually be resistant to all known antibiotic substances. This would result in uncontrollable epidemics of infectious diseases that cannot be treated. In developing countries up to 60% of the infections spread in hospitals are caused by resistant microbes. These types of hospital diseases are now also found in the community. Resistant bacteria can be transmitted between humans, but also through the consumption or handling of foodstuff with animal origin [54]. The spread of resistant bacteria from animals to humans has serious implications for the treatment of human infections since many of the antibiotics used in veterinary medicine are either identical or related to drugs used in human medicine.

2.6. Antibiotics and resistance in sewage treatment plants

The water treatment processes are dependent on the activity of microorganisms and antibiotics are therefore believed to disturb waste water treatment processes [30]. Since many antibiotics are not biodegraded in the STP, their toxicity is not eliminated. The measured concentrations of various antibiotics in STPs are in the same order of magnitude as the antibiotics' minimum inhibitory concentration (MIC_{50}), i.e. the lowest concentration at which 50% of the susceptible bacteria are inhibited [55]. Tests have shown that antibiotics can affect wastewater bacteria through both growth inhibiting and damage to the bacterial DNA. A test [31] of the influence of 18 different antibiotics on bacteria, exhibited reduced bacterial growth for ten of the drugs, e.g. thrimethoprim, in the ng/L range. Genotoxic effects are shown to be caused mainly by the fluoroquinolone antibiotics [56]. Yet another toxicity test [30] for sulfamethoxazole and ciprofloxacin on bacteria present in waste water revealed high toxicity for sulfamethoxazole, whereas ciprofloxacin had a weak but significant effect. The authors drew the conclusion that antibiotic drugs emitted into municipal sewage may affect the biological process in sewage treatment plants. The concentrations causing genotoxic effects on wastewater bacteria are in the range of 0,2-0,4 $\mu\text{g/L}$ for ciprofloxacin and 1-2 $\mu\text{g/L}$ for ofloxacin [55].

Resistant bacteria reach the wastewater plants mainly with stools excreted by humans [57]. Investigating the occurrence of resistant bacteria in Australian sewage water revealed that all bacteria tested were resistant to at least two of the six antibiotics applied. *E. coli* resistant to 16 different antibiotics were monitored in sewage water and sludge from a STP which treats water from households and from a hospital [58]. The highest resistance rates were found for tetracycline (57%), a quinolone (15%) and trimethoprim/sulfamethoxazole (13%).

It is assumed that resistant bacteria can be selected by antibiotic substances in the different processes of STPs [31]. The development of bacterial resistance due to the presence of antibiotics in STPs cannot be excluded, since the predicted average

concentrations of antibiotics in municipal sewage water are just one order of magnitude above, or in the same order of the MIC measured for these antibiotics. Exposure of bacteria to subtherapeutic antibiotic concentrations is thought to increase the pace for selection of resistant bacterial strains, but there is only scarce information about the effects from subinhibitory concentrations on environmental bacteria [5]. Horizontal gene transfer by conjugation is common in nature where the density of bacteria is high [59]. This high density is found in biofilms from wastewater systems, especially from activated sludge in STPs. It is therefore believed that horizontal transfer of genes encoding for antibacterial resistance can be induced in these biofilms. Biofilms are also generated in surface water and drinking water distribution systems. Despite the possibility of horizontal gene transfer in the STPs, studies have not been able to verify an unmistakable increase of resistance during the water cleaning process [58]. There seems to be no increase in resistance rates of *E. coli* in the course of the wastewater process. Also, the prevalence of antibiotic-resistant *Acinetobacter*, a pathogenic bacterium often found in water, in treated sewage and digested sludge were generally not significantly higher than in raw sewage and it was concluded that the wastewater treatment did not result in a selection of antimicrobial resistant bacteria [60]. Actually, in some cases the prevalence of resistant bacteria in treated sewage appeared to decrease compared to raw sewage. The frequency of resistant bacteria in sewage seems to vary depending on plant structure, bacterial strain and antibiotic drug under study, as well as the methods used to determine antimicrobial resistance.

The presence of resistant bacteria in sewage water will lead to release of these organisms into the environment [60]. Even though the number of bacteria is decreased from the inflow to the effluent by 10-1000 times, up to 100 CFU/ml¹ reach the receiving waters and thus resistant bacteria enter the environment. Generally, microorganisms accumulate in the sewage sludge and their concentration increases as the sludge is dewatered [58]. Stabilization processes decrease bacterial concentrations, but the sludge can be expected to contain resistant bacteria, depending on the stabilization method used. The resistant bacteria can then enter the environment through agricultural use of the sludge. The transfer of resistant bacteria to humans may occur via water or food if plants are watered with surface water or sewage sludge, or manure if it is used as a fertilizer [52].

2.7. Negative effects of antibiotics on non-target organisms

The release of antibiotics into the environment is not only a resistance problem. The toxicity of the substances can also hit non-target organisms, i.e. organisms other than bacteria [12]. Whilst the toxicity of antibiotics against pathogenic bacteria is well known, there is little information about their ecotoxicological effects to non-target organisms available. Also, many experiments do not take into account the possibility of additive and synergistic effects from different pharmaceuticals [61]. It is expected that a combination of drugs can exhibit both additive and synergistic influence beyond their individual effects [62]. Measured concentrations of individual substances are usually low, but the

¹ CFU: Colony forming units, a unit of measurement used in microbiology indicating the number of microorganisms present in a water sample [19].

combined concentrations of pharmaceuticals with similar modes of action can be ecotoxicologically significant [61]. It is evident that an organism is subjected to not only one, but several pharmaceutical substances, due the chemicals' common source; sewage water. Toxicity test tend to mimic acute exposure of pharmaceuticals, simulating a single release of the chemical instead of a chronic exposure, characterizing effects of a lifelong exposure [63]. The chronic exposure is more likely, since pharmaceutical and other xenobiotics are usually discharged during a long time span, but in low concentrations. In environmental risk assessments, acute testing of toxicity is generally not applicable for pharmaceuticals, since continuous exposure of the aquatic environment via STP effluents is assumed [14].

A toxicity classification [64] comparing four pharmaceutical classes has predicted antibiotics to have less relative toxicity than sex hormones, but when concerning risk-ranking relative to probability and potential severity for human and environmental health effects, the antibiotics pose the greatest threat.

An American evaluation [63] of pharmaceutical effects on *Daphnia magna*, shows that a mixture of four antibiotics, including erythromycin, lincomycin, sulfamethoxazole and trimethoprim, elicits changes in the *Daphnia* sex ratio. *Daphnia* are often used in aquatic toxicology test because of their rapid reproduction, sensitivity to the chemical environment and critical role in freshwater ecosystems by serving as an intermediate between primary producers and fish. Life history changes in *Daphnia* are believed to trigger responses in community- or even ecosystem-level. A decreased number of *Daphnia* could reduce water clarity or lead to a decline in the health of fish and other plankton-eating predators.

The influence on the growth of green algae by antimicrobial agents approved as veterinary drugs in Japan, showed that the growth inhibitory activity was very varied depending on the substance [62]. Toxicity against these organisms is considered to be of particular importance since phototrophic microalgae are the primary producers of essential nutrients in the ecosystem. It is also believed that agents showing toxicity against algae can exert similar effects on other eukaryotic organisms in soil and water, such as insects and zooplankton. The antibiotic agent erythromycin showed the strongest inhibition ($EC_{50}^2 = 0,037$ mg/L) against the algae, followed by oxytetracycline ($EC_{50} = 0,34$ mg/L). The sulfa drugs also exhibited growth inhibition to some extent ($EC_{50} = 1,53$ mg/L). The synergistic effect of combining sulfonamides with trimethoprim, which are commonly used as a combined drug, was also investigated. By adding trimethoprim, the growth inhibitory activity of the sulfonamide was significantly enhanced. The effect shown by the combination of sulfamethoxazole and trimethoprim, indicates that the simultaneous discharge of antimicrobial agents may result in higher toxicity to microorganisms in the environment than the release of the same agents individually.

An evaluation [12] of acute and chronic toxicity exerted by six antibiotics on non-target organisms was performed on five trophic levels found in surface water; bacteria, algae, rotifers, microcrustaceans and fish. The chronic test showed higher toxicity than the

² The concentration of a substance, leading to an effect for 50% of the animals exposed [35].

acute test – toxicity in the ng/L-range respectively in the mg/L-range was demonstrated. The substances showed toxic effect on all aquatic organisms tested, where the algae were found to be the most sensitive species with EC₅₀-values ranging from 0,002 to 1,44 mg/L, depending on the substance to which it was exposed. Clarithromycin and erythromycin proved to have the most potent toxic effect, i.e. the lowest EC₅₀-values, in algae, whilst oxytetracycline and sulfamethoxazole showed the highest effect on the microcrustacea. Ofloxacin was the only antibiotic showing lethal effect on fish at the highest concentration tested (1 000 mg/L). Sulfamethoxazole, ofloxacin and lincomycin also showed mutagenetic effect on two different bacterial strains. In all, the results showed that there are negative effects on non-target organisms associated with low levels of pharmaceuticals in surface waters and that the macrolids is the most harmful group of antibiotics for the aquatic environment.

Toxicity of seven fluoroquinolones has been tested on five different aquatic organisms in another study [27]. The results showed a high toxicity of the FQs to environmental bacteria, with EC₅₀-values ranging from 0,0079 mg/L for levofloxacin to 0,049 mg/L for enrofloxacin. *Lemna minor*, a water plant also called duckweed, showed EC₅₀-values in the low mg/L-range for the substances. In all, the FQs investigated in this study demonstrated limited toxicity to three of the five species tested; the bacteria, *L. minor* and *P. subcapitata*, a green algae. Fish were not generally effected by FQ-exposure even at high concentrations (2-10 mg/L), but the fish dry weight were significantly greater compared to the control group, indicating that the compounds can act as growth promoters by reducing the amount of deleterious bacteria in the fish.

The occurrence of antibiotics in manure and sewage sludge has also attracted attention, leading to evaluations of their effect on soil organisms. A German investigation [65] of soil microbial activity and microbial biomass showed that sulfonamides and tetracycline significantly reduced the number of soil bacteria. It was concluded that these substances can exert a temporary selective pressure on soil microorganisms even at environmentally relevant concentrations. The effective dose inhibiting the microbial activity ranged from total concentrations of 0,003-7,35 µg/g soil, depending on the antibiotic compound and the soil adsorption. Adverse effects on soil microbial enzymatic activities, such as phosphatase and dehydrogenase activities, have also been observed in another experiment [66]. Effects on ecologically important bacteria have been evaluated in Australia [57]. Antibiotic substances found to enter local waterways appeared to be capable of influencing biotic processes in the receiving environment. Significant depression in denitrification rates was observed for certain antibiotics, e.g. ciprofloxacin and amoxicillin. Denitrifying bacteria are important in the transformation of nitrogen in nature [35]. Soil bacteria are involved in the fixation of atmospheric nitrogen used by plants for growth [67]. Other soil organisms are involved in sulphur oxidation, organic decomposition, improvement of soil aggregation, which influences water movement and aeration. Inhibiting the activity of these organisms may therefore affect different processes in the soil ecosystem.

2.8. Transportation of pharmaceuticals into the environment

Pharmaceuticals can end up in the environment through various ways. Figure 2 is a schematic illustration over the flux of pharmaceutical substances and their metabolites into the environment, from manufacturing through waste management to biological effect. The introduction of pharmaceuticals into the environment is a result of several factors: the quantity manufactured and prescribed, the excretion efficiency of the parent compound and its' metabolites, adsorption/desorption to soil or sludge and the decomposition in sewage water [68].

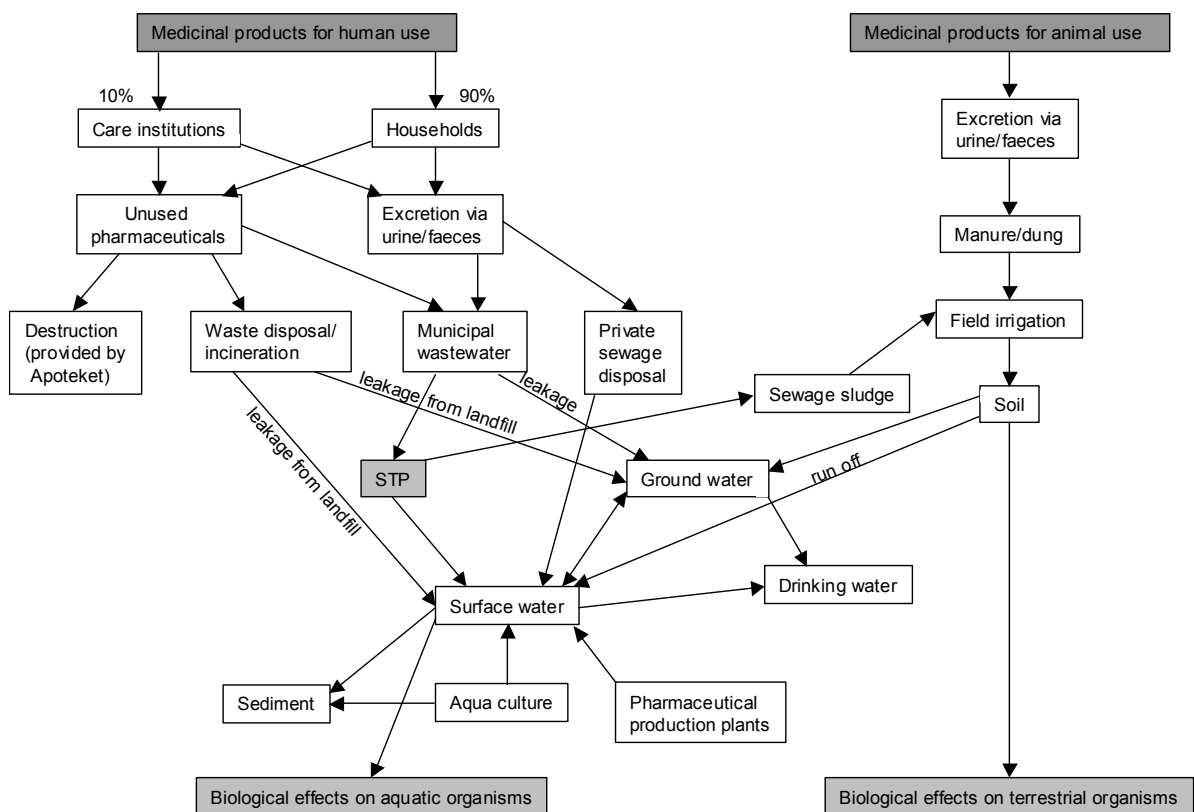


Figure 2. Fate of pharmaceuticals in the environment.

After being synthesized, the medical substances are transported and distributed to grossists and pharmacies [69]. This process is well controlled and the producers of these substances have the responsibility to take care of the chemicals in an environmentally safe way. The knowledge about what is happening with the pharmaceuticals after leaving the pharmacies is, on the other hand, rather insufficient. Approximately 90% of the pharmaceuticals distributed by Apoteket AB, are used in households, whilst the remaining 10% are used within care institutions, such as hospitals and nursing homes.

During 2004, 900 tonnes of unused pharmaceuticals were returned to Apoteket, and 42% of the Swedish population pose that they return their unused medicinal products to the

pharmacies [2]. On the other hand, an estimation of 450-1 000 tonnes of unused pharmaceuticals, worth around two billion kronor, are supposed to be thrown away or flushed down the drains every year in Sweden [70]. The returned medicine is burnt at about 850°C, to make sure that the harmful emissions are reduced to a minimum. Provided that the incineration is working, the degree of destruction of organic substances is 99% [1]. Throwing medicinal products together with the domestic waste can lead to spreading of these substances from waste landfills. Most landfills are constructed to allow a certain leakage which will lead to a discharge of persistent substances, or substances with low degradation rate into the environment. Swedish and foreign studies have, in rare cases, found pharmaceutical residues in leakage water and groundwater from landfills [1, 69].

However, pharmaceuticals and their residues are predominately distributed into the environment through human excretion, via urine and faeces [4]. The substances therefore end up in municipal sewage treatment plants, which is the main route for transportation of pharmaceuticals. Households not connected to the municipal sewage system can discharge their sewage directly to the surface water. Many of the private sewage systems are in bad shape, leading to large discharges of contaminated water. The water pipes transporting water to the sewage treatment plants can leak water, which gives the chemicals a chance to infiltrate the soil around the pipes and also reach the ground water, which can be polluted [48]. Both groundwater and surface water can be used as a source for drinking water. Since some pharmaceuticals are difficult to eliminate during the water cleaning process, they can be found in potable water.

The sewage treatment plants cannot completely purify the water from all pharmaceutical substances and metabolites [1]. Purified waters are found to contain pharmaceutical residues in concentrations up to micrograms per litre ($\mu\text{g/L}$) water. There are three possible fates of pharmaceuticals in a STP [3], either i) the substance is completely mineralized to carbon dioxide and water, or ii) the substance is lipophilic and not readily degradable, and will bind to and be retained in the sludge, or iii) the substance is metabolized to a more water soluble compound, but still persistent, and will therefore pass through the STP and end up in the recipient, polluting the surface water. Chemical substances can bind to particles in the surface water, thereafter settle and pollute the sediment. In aquaculture pharmaceuticals are distributed either via feedstuff or spread directly into the water. Through overfeeding and poor adsorption of the drugs it has been estimated that 70-80% of the drugs administered in fish farms end up in the environment. This results in contamination of surface water and sediment, where the substances can reside for a long period of time [10].

The chemicals bound to and concentrated in the sewage sludge, can end up on arable land if the sludge is used as soil improvement agent or in energy forestry [68]. The pharmaceuticals used in livestock are, after excretion, found in manure and dung, which is often used as fertilizers and therefore irrigated upon farmland. Precipitation and irrigation will lead to an infiltration of chemicals into the soil. Depending on the substances' mobility in the soil system, they may threaten the groundwater. If the chemicals are being washed away from the fields into ditches and streams, they can

eventually reach larger watercourses. When the pharmaceuticals end up in surface water, biological effects can be expected [12]. Many of the organisms living in these waters are sensitive towards pollution, and today there are many examples of organisms suffering from damage caused by pharmaceutical residues. Investigations of the soil have shown that organisms living in that environment are also negatively affected by these compounds [68].

2.9. The water cleaning process

Sewage treatment plants are constructed according to several different principles, but most large plants are composed of some common steps and cleaning procedures. The plants in Borås and Mark are similar in their construction – they are situated in the same area of Sweden and are therefore enforced to follow the same regulations for discharge. Plants situated in the colder parts of Sweden do not have the same demand for cleaning the water from nitrogen containing compounds [71]. The cleaning process described, will be a simplified and generalized picture (see Figure 3). Variations can occur between different treatment plants, depending on location, size etc, but the fundamental ideas are the same.

During the mechanical cleaning larger, solid fractions are being separated through a screen [9, 72, 73]. Sand, grit and other easily sedimented particles are thereafter separated in an aerated grit chamber. After passage through the grit chamber, remaining removable contamination is sedimented in a primary clarifier. Removal of this raw sludge to the thickener will lead to a separation of 1/3 of the oxygen demanding compounds from the sewage water, and the water soluble compounds containing nitrogen (N) and phosphorous (P) will not be removed. Mechanical treatment is therefore insufficient and regarded as a pre-treatment step, why additional cleaning is necessary.

Before the water enters the biological cleaning, a flocculation chemical can be added for the presedimentation. Heavier particles and the flocculate will then sink to the bottom of the clarifier, and reduction of both N and P will take place. In the biological cleaning step, organic material is decomposed to carbon dioxide (CO₂) and water if the process is aerobic, or to CO₂, water and methane (CH₄) if the process is anaerobic. Usually an active sludge process with biologically active microorganisms is being employed during this step. Approximately 30-50% of the organic matter is broken down to CO₂ and water and the remaining part is either transported with the outgoing water or with the sludge, as excess or return sludge. To get a sufficiently high breakdown of the biological material, a high concentration of microorganisms is required in the active sludge process. This is achieved by transporting back the main part of the sludge partitioned from the sequential sedimentation steps. 40-50% of the organic substances are transported back to the active sludge reactor as return sludge. The active sludge process is followed by a second sedimentation, where biological oxygen demanding compounds (BOD) in the excess sludge is separated as secondary sludge, which is transported to the thickener. P and N are only reduced to a moderate extent in conventional biological treatment processes. Phosphorous can be reduced both chemically and biologically, but in Scandinavia,

reduction of P principally occurs through different types of chemical precipitation. The method usually used to reduce N is based on dissimilative reduction, which initially requires a transformation of N in the sewage water to nitrate (NO_3^-) or nitrite (NO_2^-). This is achieved through denitrification, which involves oxidation of N in ammonium (NH_4^+) by autotrophe bacteria. During dissimilative reduction, nitrite and nitrate is transformed to nitrogen gas (N_2), which can be drained off into the air.

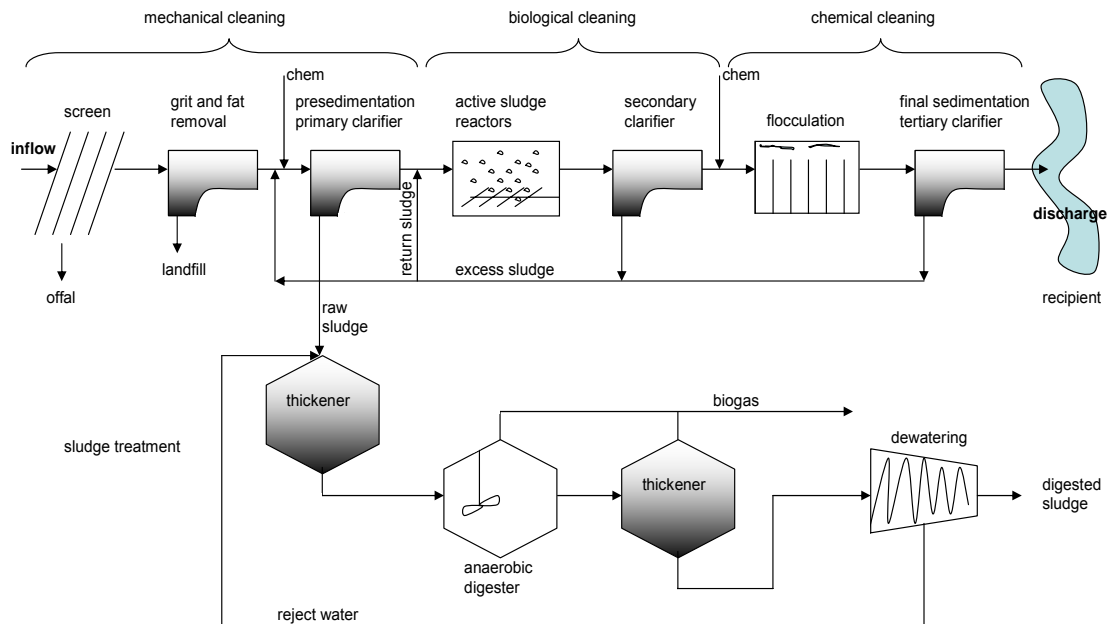


Figure 3. Schematic picture of the cleaning processes usually present in a sewage treatment plant.

In the chemical cleaning step, a flocculation chemical is added which leads to particles colliding and forming floccules. The aim of the process is to further remove or reduce phosphate and nitrogen containing compounds, BOD and suspended material. The floccules can either sink to the bottom of the basin and form tertiary sludge, or, when adding air, the particles can float to the water surface, where they are separated as sludge. After the chemical cleaning, the purified water is discharged into the recipient.

During thickening the excess water is removed from the sludge, leading to an increase in dryness. The sludge is transported to an anaerobic digester, where it is decayed and stabilized. This process occurs anaerobic at $\sim 37^\circ\text{C}$ during 15-30 days. 50% of the organic material is degraded by means of mesophile bacteria. During the anaerobic digestion the quantity of sludge is reduced, pathogens are killed and biogas, i.e. CH_4 , is generated. After the digestion, the sludge is dewatered again, before it is used on landfills, in farming as fertilizer, or is transformed to pellets.

2.9.1. Currents regulation on discharge from sewage treatment plants in Sweden

According to the Environmental Act (7th chapter, 7§) [74] sewage water should be purified or treated in such a way that inconvenience for human health or the environment does not occur. Current laws concerning discharge from sewage treatment plants [71] include threshold values for discharge of biological oxygen demand during seven days (BOD₇) and guideline values for chemical oxygen demand (COD) and total nitrogen (N_{tot}). The treatment plants are also obliged to control the discharge of BOD₇, COD, N_{tot}, total phosphorous and ammonium nitrate (NH₄NO₃) [75]. For plants dimensioned for more than 20 000 pe³, the control shall also include seven different metals, for example mercury (Hg) and copper (Cu). No regulations concerning other chemical substances are in effect.

2.9.2. The plants in Mark and Borås

In Mark's municipality there are several different types of sewage treatment plants, seven smaller plants and one large in Skene, serving the population centre of the municipality around Kinna and Skene [72, 76]. Sludge from the smaller wastewater plants and from private septic tanks is transported to Skene STP. Industrial wastewater from several textile industries is also conducted to the plant. Borås municipality operate eight smaller STPs in addition to the large plant, Gässlösa [73, 77]. Sludge from septic tanks, other municipal STPs and from certain industries, e.g. an abattoir, is transported to Gässlösa. Wastewater from various industries, such as textile and chemical industries, is also transported to the plant. Basic data on the STPs are found in Table 4. In all there are 21 municipal STPs with their discharge into Viskan or its' catchment, where the plants in Skene and Borås are the largest [78].

Table 4. *Technical data for the sewage treatment plants in Mark's and Borås' municipalities.*

Municipality	STP	pe load 2004	Hospitals connected to STP	Average daily flow 2004 [m ³ /d]
Mark	Skene	10 300	1	11 791
Borås	Gässlösa	110 000	1	48 963
			Σ 530 beds	

³ A person equivalent is the average amount of pollutants in sewage water derived from one person per day. BOD₇ is the biochemical oxygen consumption in water during seven days. pe is an indirect measure of the amount of organic substances in water and in Sweden defined as a BOD₇-load of 70g/day [33].

2.10. The recipient – Viskan River

Viskan river has its' source in the lake Tolken and runs through the municipalities of Ulricehamn, Borås, Mark and Varberg, in Västra Götaland in the southern part of Sweden before it, 100 km later, reaches Kattegatt [79]. Viskan's catchment is 2201 km² and the number of inhabitants in the area is estimated to 140 000 [80]. The drainage area is mainly constituted by woodland (75%) and agricultural land (14%) [78]. The area around Viskan is one of the regions with the highest precipitation in southern Sweden. From January until April the amount of water in Viskan is usually relative high because of the melting away of snow.

Viskan river was, as early as the second half of the 19th century, so burdened with pollution, that the water quality was markedly poor [81]. At this time, the building up of the massive textile industry started along Viskan. The river represented an important source of energy and raw material for the industries, as well as the recipient for the waste produced by the industries. During the 20th century, the situation deteriorated and in the fifties toxic substances caused obvious negative effects upon the river, including death of fish. The development and expansion of sewage treatment plants along Viskan during the seventies lead to large improvements of the environment.

In 1961 Viskan Vattenvårdsförbund, a non-profit organization for companies, municipalities, the county council and various associations, was founded to supervise and inspect the environmental quality of the river [79]. The organization is responsible for carrying out annual recipient inspections – including water chemistry, benthic fauna, fish and sediment. Today it is still possible to find high concentrations of heavy metals, chlorinated compounds, brominated flame retardants and dioxin in the sediments of Viskan [82]. The river have since long been an important stream for the spawning of salmons and parts of Viskan's long valley are now considered national interest for nature conservation and environment protection, recreation and outdoor life, such as angling for eel and salmon [79]. It is therefore important to keep Viskan and its surroundings in a good condition.

2.11. Environmental risk assessments

Risk is a term used to denote the probability that a harmful consequence will occur as a result of an action – it is a function of both hazard and exposure [83, 84]. For risk to occur there must be a source of risk, a hazard, and an exposure to this hazard. The hazard concerned in an environmental risk assessment (ERA), refers to the potential adverse effect posed by a chemical substance. Risk assessment is the process by which one attempts to evaluate and predict the likelihood and extent of harm that may result from

an, in this case, environmental hazard. An ERA shall provide essential information about the severity and extent of specific environmental problems.

There is a large number of guidelines for performing environmental risk assessments, but an ERA typically involves answering a few questions concerning the hazard's properties, probability and magnitude of exposure, probability of harm, significance of risk and possible uncertainties [84]. The ERAs are preceded by a vast assembly of information, both through collecting literature data and through actual experimental assessments [14]. Performing an ERA is a step-wise procedure that may be terminated when sufficient data are available, to either indicate that the substance is unlikely to represent a risk to the environment, or to identify and adequately characterize the potential risk.

2.11.1. The European Medicines Agency (EMA) risk assessment

A draft for a guideline on the ERA of medicinal products for human use has been published by EMA [14]. The guideline relates to the environmental risks arising from use, storage and disposal of medicinal products. Though, the guideline is not applicable for risk evaluation concerning synthesis or manufacture of these products. According to the guideline, the products that exhibit potential risk to the environment should be accompanied by appropriate precautionary and safety measures. These measures should be observed when administered to patients and/or for disposal of waste products. Regardless of the ERA's result, the benefits for patients have precedence over the possible environmental risk caused by the pharmaceutical. An effective medicine will therefore never be put out of market because of its' negative environmental effects.

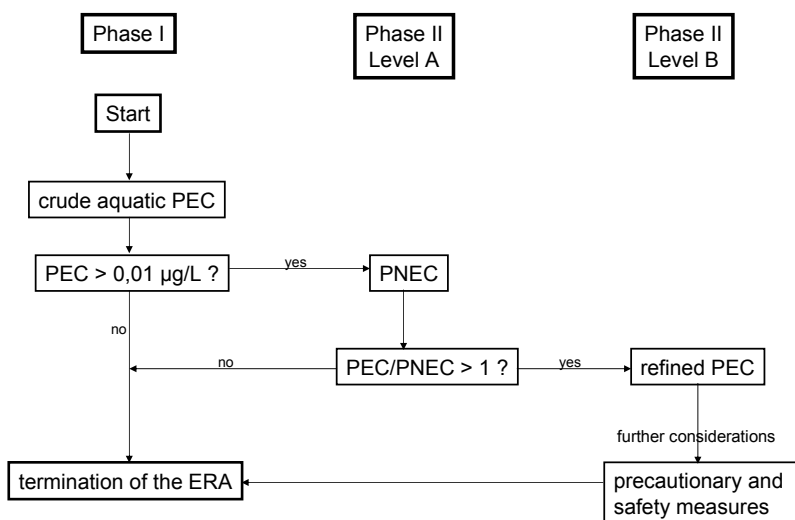


Figure 4. Flow chart of the EMA environmental risk assessment work process.

The EMA risk assessment consists of two levels, where Phase I is focused on the environmental exposure to the active substance and/or its metabolites [14, 15]. This level includes calculation of a crude aquatic predicted environmental concentration (PEC_{crude}).

Usually only the aquatic environment is considered in the ERA, since most pharmaceuticals end up in that compartment. When calculating the PEC, one assumes, among other things, that the sewage system is the main route of entry of the substance into the surface water and that there occurs neither biodegradation in the STP, nor metabolism in the patient, illustrating a worst-case scenario. If the PEC_{crude} is below 0,01 $\mu\text{g/L}$, it may be assumed that the pharmaceutical is unlikely to constitute risk for the environment. If, on the other hand, the value exceeds 0,01 $\mu\text{g/L}$, phase II of the ERA should be performed. In the second phase, data about the substance's physical/chemical, pharmacological and toxicological properties are appraised in relation to the extent of exposure of the environment. Phase II is divided into two sublevels – level A begins with an evaluation of the fate and effects of the medicinal product. This level involves information on the product's toxicity to environmental organisms, degradability, biodegradation in water and potential for bioaccumulation. The predicted no effect concentration (PNEC) is evaluated and compared with the PEC. PNEC represents the environmental level at which no adverse effect on the ecosystem is expected. If no risk is detected on this level and if no bioaccumulation is appointed, the assessment will be terminated. If, on the other hand, the PEC/PNEC ratio is above 1, i.e. risk cannot be excluded, further evaluation is needed and the assessment continues with level B. In this step, the PEC is refined by taking metabolism and transformation during environmental distribution into account. Data concerning excretion of substance, adsorption to sewage sludge and biodegradation of substance are also included when recalculating the PEC. A new PEC/PNEC ratio is now calculated and if environmental risk cannot be excluded on this level, precautionary and safety measures are necessary. These may include restricted clinical use or product labelling for storage, disposal and patient use [15].

2.12. Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS)

Mass spectrometry is widely used for detection in chromatography to provide both qualitative and quantitative information [85]. Liquid chromatography (LC) combined with mass spectrometry (MS), allows separation of complex mixtures of non-volatile compounds before introduction to the mass spectrometer. It is used extensively for compounds that have a high molecular weight or are too sensitive to heat to be analyzed by gas chromatography (GC). LC-tandem MS is usually employed for polar pharmaceuticals, since there is no need for derivatization. Derivatization is the process of chemical modification to a compound which has properties more suitable for analysis by GC, such as achieving adequate volatility or stability.

2.12.1. Liquid chromatography

The mobile phase in LC refers to the solvent being continuously applied to the column. The stationary phase is the solid support contained within the column over which the mobile phase flows. The mobile phase acts as a carrier for the sample solution, which is injected into the column via an injection valve with a sample loop. The sample, usually dissolved in the mobile phase before injection, is first drawn into a syringe and injected

into the loop, thereafter into the stream of the mobile phase. The chemical interactions of the mobile and stationary phase and the analytes, determine the degree of migration and separation of components contained in the sample. Compounds showing stronger interactions with the mobile phase than with the stationary phase, elute from the column faster and will therefore have a shorter retention time, and vice versa. Both the mobile and the stationary phase can be altered in order to manipulate the interactions of the sample elements and the phases.

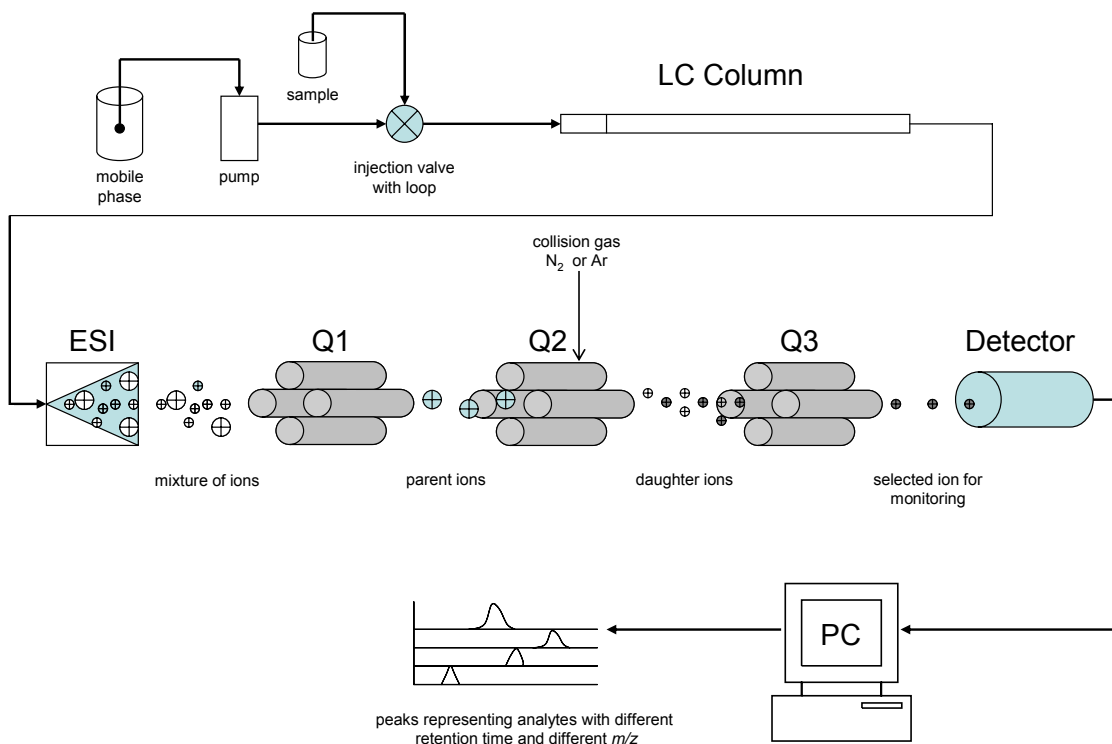


Figure 5. General principles of the LC-ESI-MS/MS setup.

2.12.2. Electrospray ionization

Electrospray is one of the commonly used methods for introducing analytes from LC into a mass spectrometer, facilitating the ability to analyse compounds directly from aqueous solutions [86, 87]. The liquid from the column creates a huge volume of gas when it is vaporized at the interface between the LC and the MS. Most of this gas and the non-volatile mobile-phase additives from the LC must be removed prior to ion separation in the MS.

The analytes are introduced to the electrospray source in solution from the LC. The liquid is passed through a nozzle, at the end of which a potential is applied. Soon the liquid reaches a critical point, at which the potential applied is high enough to disperse the solution and at the tip of the nozzle it blows apart into a cloud of tiny, highly charged droplets. Electrospray ejects pre-existing ions from the solution into the gas phase but does not create new ions. The solvent will evaporate away, shrinking the droplet size and

increasing the charge concentration at the droplet's surface. Eventually the droplet's surface tension cannot hold the droplet together and it explodes, forming smaller, lower charged droplets. These explosions are repeated until individually charged analyte ions are formed.

2.12.3. Tandem mass spectrometry

Selected reaction monitoring (SRM) is used to increase the selectivity of MS for individual analytes and improve the sensitivity by decreasing the background noise. The signal-to-noise ratio can markedly be increased by using the SRM mode, here illustrated with a triple quadrupole mass spectrometer in Figure 5. A quadrupole mass analyser consists of four parallel rods that have constant voltage and alternating radiofrequency potentials applied to them. The ions are passed along the middle of the quadrupoles, from the ion source, i.e. the ESI, toward the detector. The ions' movement pattern will depend on the electric fields so that only ions of a particular mass-to-charge ratio, also denoted m/z , will be in resonance and thus pass through to the detector. Other ions collide with the rods of the quadrupole and are lost before they reach the detector. The applied voltage can be varied to bring ions of different m/z into focus of the detector and thus build up a mass spectrum. In a triple quadrupole mass spectrometer a mixture of ions enter the first quadrupole Q1, which passes just the one selected parent ion to the second stage, Q2, which is also called the collision cell. In Q2, the parent ion collides with a gas, usually N₂ or Ar, breaking them into fragments called daughter ions. The last quadrupole, Q3, only allows specific daughter ions to pass through the detector, giving a mass spectrum of the analytes chosen to be detected.

3. Method

3.1. Sampling strategy

3.1.1. Sewage water

Incoming and outgoing water from the sewage treatment plants were collected for seven consecutive days, except for one sample from Gässlösa. Samples from Skene STP were collected on the 8th-14th March in 2005. A problem with the sampling equipment made it impossible to collect water on 15th of March at Gässlösa STP, why a sample for the 16th of March was used instead. Sampling from Gässlösa began on the 9th of March until the 14th, and also on the 16th. The 24-hour-samples were mixed proportionally to the water flow in the STP and these samples were thereafter pooled proportionally to the flow during the week of interest. The seven-day-samples were kept frozen until clean up began.

3.1.2. Recipient water

The sampling in the recipient was performed during four consecutive days in mid-March (14-17 March) 2005. The recipient water samples were collected with a Ruttner water sampler, equipped with an internal thermometer for registration of the in situ temperature.

The samples were collected mainly by lowering the Ruttner from bridges into the main stream of the river. Two of the samples (sampling points B and I, see Figure 6) were obtained using a water collector for inaccessible open waters. The collector can be elongated up to approximately three metres, only allowing sampling close to the river bank and not in the main stream of Viskan.

Water temperature at the river sampling points ranged from 1 to 2°C. The river was partially covered with ice and the water flow during the sampling period fluctuated from slightly low to normal, compared to the mean annual flow [88]. The samples were collected in 1 L plastic bottles and stored in a freezer until analysis.

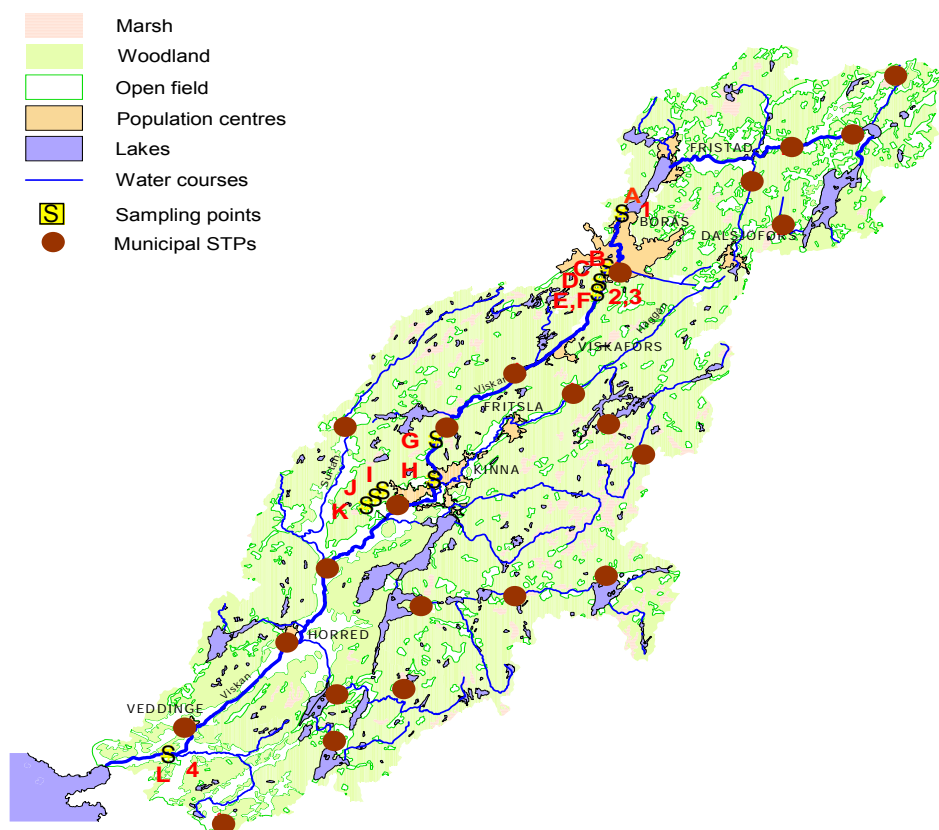


Figure 6. Map of Viskan's catchment area, with water (A-L) and sediment (1-4) sampling points [89].

The location of the water sampling points in Viskan was mainly dependent on where the bridges downstream the treatment plants are situated, since the steep river banks of Viskan make it difficult to reach the water without appropriate means, such as a boat or a canoe. According to a previously performed sampling and analysis of antibiotics in recipient water, some antibiotics can be detected, though in very low concentrations, 500 metres from the outlet of a treatment plant [57]. Regarding these results, the sampling

point should preferably not be located further downstream than 500-1 000 meters from the STP, where possible. This requirement could not be fulfilled for all sampling points. Furthermore, sample points should preferably not be situated too close to the plant's outlet, since the sewage water need time to mix with the recipient water. Incomplete mixing can give large variations in xenobiotic concentrations for the recipient water. In all, 12 recipient water samples were collected. The location of the sampling points can be seen in Figure 6 (marked with A – L, see also appendix 1).

3.1.3. Recipient sediment

Sampling of sediment has proved to be difficult in running waters. Golder Associates have been commissioned by the County Administrative Board of Västra Götaland to outline the spread of sedimentation areas and the occurrence of organic and inorganic toxicants in the sediments of Viskan. According to their report [82], the prerequisite for accumulation of fine and lightweight particles, and therefore also xenobiotics, are fair upstream Kinna, where several large and small lakes constitute larger, coherent sedimentation areas. Downstream Kinna there are only limited areas, where certain temporary accumulation of fine particles can occur. Fine particles and bound substances, are transported with the water of Viskan to its' outlet in Kattegatt. Golder's conclusion is that it can be difficult to find accumulated sediment with relevant organic content for analysis in Viskan. According to Ulf Ericsson at Medins Biologi AB [90], sediment samples should preferably be collected in lakes or expanded river areas. Sediment can also be found near reed or other vegetation. River areas where the water flow is reduced give higher chance in finding sediment containing organic material, whereas fast flowing streams are not worth examining for sediment with organic content [90, 91]. Sediment sampling points can be located further downstream from the STP than water sampling points, since particle-bound chemicals need a certain distance to settle and incorporate with the sediment. Totally four sediments samples were collected – their location can be seen in Figure 6 (marked with 1-4, see also Appendix 1).



Figure 7. From left to right: Collecting water with Ruttner at Åsbro sampling point, Ruttner water sampler [92], Ekman grab sampler [92] and water collector for open waters [92].

For collection of the sediment, an Ekman grab sampler was used. Several points were tested for sediment, but only four relevant sediment samples could be collected. The samples were stored in plastic containers and kept frozen until analysis.

3.2. Chemicals and standards

Enrofloxacin, norfloxacin, ofloxacin and divaveridine were purchased from Sigma Aldrich (Stockholm, Sweden). Ciprofloxacin and sulfamethazine were bought from ICN Biochemicals/Irvine, CA), whilst sulfamethoxazole and trimethoprim were obtained from Duchefa (Haarlem, The Netherlands). Formic acid and methanol (HPLC-grade) were purchased from J.T. Baker (Deventer, The Netherlands), and acetonitrile (HPLC-grade) was bought from Fischer Chemicals (Zurich, Switzerland). Triethylamine (TEA), dipotassium hydrogen phosphate, acetic acid and sulphuric acid were obtained from Merck (Darmstadt, Germany). The purified water (resistivity 18,2 M Ω ·cm) used was prepared by passing water through an ELGA Maxima HPLC ultrapure water system (ELGA, High Wycombe Bucks, England), equipped with a UV radiation source. All other chemicals were of analytical grade.

Standard stock solutions of each antibiotic were prepared with water, except from sulfamethoxazole and sulfamethazine (IS) which were dissolved in methanol. By adding a maximum of 5% (v/v) acetic acid to the standards, the solubility of enrofloxacin (IS), norfloxacin, ofloxacin, diaveridine (IS) and trimethoprim was enhanced. Mixed standard solutions of both IS and analytes were prepared from these standard stock solutions and water. The IS-mix solution was diluted once more to be used in the water samples. All standard solutions were kept dark in the freezer at -18°C when not used. The analyte- and IS-mixes were used for preparing calibration standard solutions. The calibration standard solutions were diluted in water with 5% (v/v) acetonitrile and the same volume of IS added to the samples was added to these solutions. The calibration solutions were prepared the same day as experiments or quantification was performed.

3.3. Sample preparation

3.3.1. Water samples

The extraction method used for the water samples is based on a previous study, developed and validated for determination of antibiotics in sewage water [8, 93] and surface waters [44].

The water samples were filtrated through 0,45 μ m MF-membrane filters (Millipore, Sundbyberg, Sweden), followed by an adjustment of pH to 3, using sulphuric acid. An ENV+ column (600 mL, 200 mg) was employed for the solid phase extraction (SPE). The columns were conditioned prior to sample extraction using 5 mL methanol, 5 mL 50% (v/v) methanol in water and 5 mL water at pH 3 respectively. To each sample, 50 μ L of internal standard (IS) mix was added. The volume corresponds to an amount of ~ 500 ng of each compound, i.e. enrofloxacin, sulfamethazine and diaveridine. 800 mL of the

recipient water samples, and 600 mL of the sewage water were extracted at a flowrate of $3 \text{ mL} \cdot \text{min}^{-1}$. The columns were then washed with 5 mL water (pH 3) before being dried with air during 2-3 minutes. Elution of the target compounds was obtained using 7 mL triethylamine (TEA) (5% v/v) in methanol. The eluates were evaporated to a volume of approximately 20 μL , using air and thereafter dissolved in water/acetonitrile 95/5 (v/v) to a final volume of 400 μL for the recipient samples and 600 μL for the sewage samples, giving a concentration factor of approximately 2 000 and 1 000 respectively.

3.3.2. Sediment samples

The experimental procedure employed for the sediment samples is based on a method originally developed for determination of antibiotics in sewage sludge samples [8]. The frozen sediment was first thawed, then dried in room temperature over night to reduce the water content. 2 g of each sediment sample were thereafter weighted into a centrifugal glass tube. A McIlvain citrate-phosphate buffer (pH 6), containing 0,2 M K_2HPO_4 (I) and 0,1 M citric acid 63/37 (v/v), was prepared and added to the glass tubes. The solvent-sediment mixtures were thoroughly hand shaken before being ultrasonicated for 30 min and then centrifuged for 10 min at 4 800 rpm. The supernatants were removed and placed in 30 mL glass vials. 10 mL of 5% TEA in methanol/water (25/75, v/v) were added to the residue and further extracted as described above. The two supernatants were combined and the internal standard-mix was added prior to analysis. The volume of IS added corresponds to approximately 1,5 μg each of enrofloxacin, sulfamethazine and diaverdine.

To determine the dry weight, 4 g of each dried sediment sample were placed in an oven at 105°C for 24 h and then weighted. These results should be used for dry weight correction of the results. The percentage weight loss when sediment is dried is determined by its composition. Generally sediments with a high organic content weigh less when dried than an equivalent wet weight of sediment with a high mineral content. To determine the loss on ignition (LOI), the samples were placed in the oven at 550°C for 2,5 h and then weighted. The percentage weight lost on ignition gives a crude measure of the organic content of the sediment [94]. Generally, percentage loss-on-ignition-values show an inverse relationship with percentage dry weight values.

Blank samples were prepared for both water and sediment analysis and analyzed simultaneously as the samples to correct for possible contamination that may occur during the extraction and analysis.

3.4. Identification and quantification

Analyses of the samples were, according to previous studies of the target chemicals in sewage water and sludge [8, 93], performed using LC-ESI-MS/MS. Identification of the analytes is based on their different chromatographic retention times in the column and the mass-to-charge (m/z) values of the parent and daughter ion for each substance. The

analytes were quantified by selected reaction monitoring (SRM), where the most abundant daughter ion is used in each case.

Table 5. Monitored ions and water limits of quantification (LOQ) of the analytes and their respective internal standards [8].

Substance	m/z parent ion	collision energy [%]	m/z daughter ion	water LoQ ^a [ng/L]
enrofloxacin (IS)	360,1 [M + H] ⁺	28	316,2 [M – CO ₂ + H] ⁺	
norfloxacin	320,0 [M + H] ⁺	27	276,2 [M – CO ₂ + H] ⁺	7
ofloxacin	362,1 [M + H] ⁺	26	318,1 [M – CO ₂ + H] ⁺	6
ciprofloxacin	332,1 [M + H] ⁺	30	288,2 [M – CO ₂ + H] ⁺	6
sulfamethazine (IS)	279,0 [M + H] ⁺	28	203,8	
sulfamethoxazole	254,0 [M + H] ⁺	29	187,9	80
diaveridine (IS)	261,2 [M + H] ⁺	37	123,1	
trimethoprim	291,0 [M + H] ⁺	38	230,1 [M - 2CH + H] ⁺	8

^a Limit of quantification (LoQ) is the smallest analyte concentration for which a method has been validated with specified accuracy and precision [85]. Limit of quantification when water samples are concentrated 1 000 times. LoQ is divided by 2 when the samples are concentrated 2 000 times.

3.4.1. Liquid chromatography-mass spectrometry

10 µL of each sample extract and calibration solution were injected by use of an autoinjector into the guard column, followed by the analytical column. The analytes were chromatographically separated using a 15-min linear gradient of 95-99% H₂O balanced with acetonitrile (ACN), both containing 0,1% (v/v) formic acid, at a pump generated flow rate of 0,8 mL·min⁻¹. The mass spectrometer was used with an electrospray ion source in positive ion mode. The MS/MS parameters were optimized semiautomatically for each substance by use of LCQ Duo internal software, whilst the collision energy used to produce daughter ions was manually optimized. All the instruments used for analysis are presented in Table 6.

Table 6. *Technical data for the LC-MS/MS devices.*

Instrument	Dimensions	Settings	Manufacturer
AS 3000 Autoinjector			Thermo Finnigan, San Jose, CA, USA
YMC Hydrosphere guard column	10 x 4 mm interior diameter (i.d.) 5 µm particle size		YMC Inc., Wilmington, NC, USA
YMC Hydrosphere C18 Analytical column	150 x 4,6 mm i.d. 5 µm particle size		YMC Inc., Wilmington, NC, USA
P4000 HPLC Pump		Flow rate: 0,8mL·min ⁻¹ at 25°C	Thermo Finnigan, San Jose, CA, USA
Mass spectrometer LCQ Duo Ion Trap		Source voltage: constant 6,0 kV Heated capillary temp: 250°C	Thermo Finnigan, San Jose, CA, USA

3.4.2. Internal standards, identification and quantification

Quantification of the analytes in both water and sediment samples was obtained by using the internal standard calibration method. For the water samples, a known amount of all internal standards was added prior to SPE and the same amount of IS was also added to the calibration solutions. Analyte/IS ratios were calculated and no correction for recovery was performed. A five-point calibration curve in H₂O/ACN (95/5 v/v) was used with concentrations ranging from 0,015 ng/mL to 900 ng/mL of the analyte.

For the sediment samples, the IS was added before injection into the LC-MS to allow compensation to be made for variations that might occur during analysis, such as varying injection volumes and ionization efficiencies. Thus analyte/IS ratios were corrected for recovery obtained in the experiments made elsewhere [8]. A five-point calibration curve in H₂O/ACN (95/5 v/v) was used with concentrations ranging from 20 ng/mL to 600 ng/mL of the analyte.

3.5. Environmental risk assessment

The predicted environmental concentrations (PECs) were calculated according to the EMEA guideline [14]. No ecotoxicity tests were carried out during this project. Values of the (no) effect concentrations were therefore found in literature. According to the guideline, a standard long-term (i.e. chronic) toxicity test set on fish, daphnia and algae should be employed to determine the PNEC_{WATER}. The lowest value of the respective toxicity test should be used for risk characterisation in the ERA. For that reason, the lowest NOEC⁴/EC₅₀/LC₅₀⁵-values found in literature, were used for calculating the PNECs.

⁴ The concentration of a substance, giving no effect on the animals exposed [51].

3.6. Calculations

3.6.1. Concentrations of analytes in the samples

An internal standard is a known amount of a compound, similar but not identical to the analytes. Internal standards are added to the sample to correct for analyte losses during extraction and compensate for varying instrument responses between runs. Any loss of analyte during the extraction and analysis will be compensated for the same amount of standard lost – the ratio between the standard and the analyte remains the same. The concentration of the analyte is then measured relative to that of the internal standard. The use of calibration curves is based on the correlation of analyte and internal standard ratios in both sample and calibration standards. By using the ratios, the volumes of the sample and the standards become irrelevant, only their internal relationship is of concern.

The amount of analyte in the sample is calculated according to the following formula:

$$m_{analyte,sample} = \frac{\left(\frac{Area_{analyte,sample}}{Area_{IS,sample}} \right)}{slope_{calibrationcurve}}$$

This equation is based on the equation of the straight line

$$y = kx + m$$

where y is the ratio of the analyte and IS areas

k is the slope of the calibration curve of the particular analyte

and m is the intercept of the calibration curve with the y -axis, here set to 0, since the curve passes through origin.

3.6.2. Mass flows

The daily mass flow of the detected antibiotics was calculated using the average daily flow rate of sewage water in the STPs during 2004 (see Table 4) and the measured concentrations for each antibiotic compound. Influent and effluent volumes were supposed to be equal.

⁵ The lethal dose of a compound for 50% of animals exposed [51].

3.6.3. Predicted environmental concentration (PEC) and predicted no effect concentration (PNEC)

A PEC for the aquatic compartment, or rather for the local surface water, is calculated using the following formula [14];

$$PEC = \frac{A \times (100 - R)}{365 \times P \times V \times D \times 100} \quad [\mu\text{g/L}] \quad \text{where}$$

A is the amount active substance used per year in the test geographic area [mass/year].

R is the removal rate due to biodegradation or loss through adsorption, volatilisation, hydro- or photolysis [%]. Set to zero when information on removal in the STP is missing, or when assessing worst-case scenarios, i.e. the crude PEC.

P is the number of inhabitants in the geographic area.

V is the volume of wastewater used per inhabitant per day [L]. The default value is set to 200 L·inhabitants⁻¹·day⁻¹ in the European Union. The amount of water used per person in Sweden is also estimated to 200 L·d⁻¹ [95].

D is the dilution factor of sewage water by surface water. Default value is set to 10 for the EU. The dilution of sewage water in Viskan seems to be in the same range as the default value. Comparing the mean annual water flow at Jössabron (sampling point C, see Figure 6), with the annual flow at Gässlösa STP gives a dilution factor of 11,6 [96].

100 is the compensation for the percentage values for removal.

For the PEC_{crude} a scenario is established under the following assumptions; all sold pharmaceuticals are used in the same year, the pharmaceuticals are released into the sewage system, there is neither elimination in man nor the sewage system and the use pattern is evenly distributed temporally and spatially, since no regional consumption data was available. The number of inhabitants in the region is estimated to 140 000, which corresponded to approximately 1,55% of the total number of inhabitants in Sweden at the end of year 2004 [80]. The amounts of the target antibiotics used are thereafter corrected for the region, by assuming that 1,55% of the amounts of these substances sold in Sweden, are used within the area.

For the refined PEC, removal is set to an average value of 82% for the fluoroquinolones in sewage systems [44], 50% for sulfamethoxazole [97, 98] and 0% for trimethoprim, since it is not removed during the processes in STPs [8]. The amount active substance used is corrected for metabolism in man. Metabolism for each compound can be found in Table 1.

When calculating PNEC, the lowest 50%-effect concentrations, i.e. EC₅₀ or LC₅₀, or the lowest no-observed-effect concentration (NOEC) found, can be used. The chosen concentration should then be divided by an uncertainty factor of 1000 to derive the PNEC [14, 15, 17]. This factor is an expression of the degree of uncertainty in the extrapolation from the test data on a limited number of species to the real environment. This factor accounts for inter- and intra-species variability in sensitivity as well as extrapolation from acute to chronic toxicity.

PNEC is derived by using:

$$PNEC = \frac{\text{lowest}EC_{50} / LC_{50} / NOEC}{1000}$$

In this study only the chronic PNEC-values found in literature was used. The PEC/PNEC ratio is thereafter calculated to conclude whether the pharmaceutical is likely to present a risk for the environment or not.

4. Results and discussion

4.1. Concentrations of the antibiotics in sewage waters

Table 7 shows the detected concentrations of the target antibiotics in samples from the sewage treatment plants. Sulfamethoxazole was below its' LoQ in all sewage samples, and ofloxacin was not detected in Gässlösa STP. Ofloxacin is an antibiotic prescribed only locally and in smaller amounts than the other FQs [2], and therefore usually not detected in all sewage waters [8, 34]. It is therefore not surprising to detect it in Skene but not in Gässlösa. Since no regional consumption data was available, it is not possible to see if ofloxacin is used more extensively in this region than in other regions in Sweden. Sulfamethoxazole is usually more frequently prescribed than ofloxacin, but was still not detected in the sewage waters. This may be due to the drug's higher metabolism in the human body [24], leading to lower concentrations of the parent compound in sewage water. Also, sulfamethoxazole has a tenfold higher LoQ than the other substances, which means that it may only be detected if it is present in relatively high concentrations in the sewage water.

All FQ concentrations were higher in Skene STP than in Gässlösa, which may indicate a higher prescription rate of these compounds in the Skene area. A possible explanation is also that the hospital sewage water from Skene is less diluted in the treatment plant than the water from Borås' hospital. Hospital waters usually hold higher concentrations of antibiotics, and other pharmaceuticals, than water exclusively from households [28]. There is no available data on the proportion of hospital wastewater in the STPs.

Concentrations of the detected FQs are much higher in raw sewage than in the final effluent, showing that they are totally removed from the water before being discharged into the recipient. Removal of these compounds during the water cleaning process is due to sorption to sewage sludge [8, 99]. Since the average removal rate of fluoroquinolones in STPs is estimated to 82% [44], the results from these experiments are reasonable. Because the water samples were filtered prior to extraction, and suspended particles were not analyzed, the observed removal accounts only for FQs occurring in the dissolved fraction of the final effluent. According to other studies [8, 99], trimethoprim is assumed to be practically unaffected by the water treatment process and will therefore be found at similar concentrations in both raw sewage and final effluent water. For that reason, the concentrations found during this analysis are rational.

Table 7. Concentrations of the antibiotics in influent and effluent sewage water, corrected for the blank.

Sampling point	Gässlösa	Gässlösa	Skene	Skene
	raw sewage	final effluent	raw sewage	final effluent
	[ng/L]	[ng/L]	[ng/L]	[ng/L]
Substance				
Norfloxacin	6	< LoQ	149	< LoQ
Ofloxacin	< LoQ ^a	< LoQ	19	< LoQ
Ciprofloxacin	100	< LoQ	473	< LoQ
Sulfamethoxazole	< LoQ	< LoQ	< LoQ	< LoQ
Trimethoprim	166	128	112	121

^a< LoQ: below limit of quantification.

When comparing these results with detected concentrations from other investigations (see Table 3) one can see that all levels found in this analysis are reasonable. The concentration of norfloxacin in the raw sewage at Gässlösa is rather low, but may be due to low consumption of this compound in that area. The presence of sulfamethoxazole has been investigated in several Swedish STPs, but could not be detected in all plants, neither in the raw sewage nor the in effluent [8]. The concentration of ciprofloxacin in Skene raw sewage is rather high, but not unreasonable. In all, the concentrations detected in this experiment are fairly low compared to other analyses performed in Sweden and internationally.

4.2. Mass flows of the antibiotics in the sewage treatment plants

The daily mass flows of the antibiotics' detected at Skene and Gässlösa STP are presented in Figure 8. The mass flows for the FQs clearly show that they are effectively removed from the sewage water during the purification processes in a STP. One can also see that the amount of FQs entering the STP is greater in Skene than in Gässlösa. This is surprising since Skene STP serve a smaller number of people compared with Gässlösa. This indicates that a higher amount of these substances are used per inhabitant in Mark's municipality in comparison to Borås'.

During one year, six kilos of trimethoprim will be released from Skene and Gässlösa STPs altogether. It can be assumed that quite a number of kilos of other antibiotic substances and xenobiotics are also discharged from STPs every year into the recipient. The discharged amounts of antibiotics may seem small, but one does not know the adverse consequences of these compounds together with other substances with possible ecotoxicological effects. Small amounts of many different compounds released into nature may lead to effects that cannot be derived from a single substance. The amounts released from sewage treatment plants should therefore not be neglected.

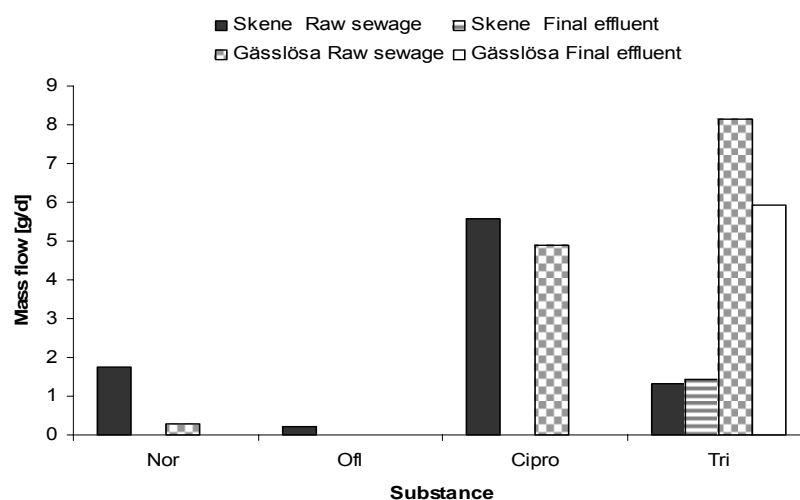


Figure 8. Mass flows of the antibiotics in raw sewage and final effluent at Skene and Gässlösa STP expressed in grams per day [g/d].

4.3. Occurrence of the antibiotics in the environmental samples

4.3.1. Water samples

All antibiotics were below their respective LoQ in the recipient samples. Though, the concentrations of trimethoprim were between its detection limit and LoQ in four recipient samples (sampling points B, C, D and G – see map in Figure 6). Theoretically, trimethoprim should be the substance with the highest concentrations in the recipient, due to its persistence in water. The concentration of this substance in the outgoing water from the STPs was over 100 ng/L (see Table 7) and the final effluent is diluted around ten times [96], therefore the concentration of trimethoprim should be around 10 ng/L in the recipient, i.e. above the LoQ for this substance. This is not the fact, which may be due to a higher dilution. The time of the sample collection coincided with a period of high precipitation and the melting of snow, which may have increased the water flow of Viskan and therefore a higher dilution of the outgoing sewage water could be expected.

FQs are predominantly sorbed to particles found in the effluent water. The water samples were filtered previous to solid-phase extraction, and suspended particles were not analysed. It is likely that most of the FQs in the final effluent water were filtered away and therefore not detected in the outgoing water. The amount of suspended solids in the final effluent is very low, which means that a small amount of FQs will reach the recipient sorbed to the particles. However, the concentrations of FQs in final effluent water with particles will be very low, due to their entrapment in sewage sludge.

In addition to the small amounts of antibiotics discharged with the final effluent, the large dilution factor of the sewage water when it reaches the recipient, will give rise to very low concentrations of the antibiotics in the recipient. It is therefore most likely that all

antibiotics are below their LoQ in the recipient. However, this does not mean that there are not any antibiotic substances present in the water – it only means that the concentrations are too low to measure. The mass flows represent a clear illustration of the quantities of antibiotic released into the recipient every day.

The concentration of FQs can be reduced in surface water either through phototransformation and sorption to particles [44]. Other processes, such as biodegradation and gas exchange, are not relevant since the substances are assumed to be relatively slowly degraded, also the elimination by gas exchange is considered unlikely because the FQs are ionic compounds with relatively high molecular weight and therefore have a low tendency to volatilize. The same argument is supposed to hold to some extent for trimethoprim and sulfamethoxazole. They are also not readily biodegraded and have relatively high molecular weight. On the other hand, trimethoprim and sulfamethoxazole have not as strong tendency for adsorption on particulate matter as the fluoroquinolones, but sulfamethoxazole tend to be more easily phototransformed than the FQs [29]. In all, phototransformation is supposed to be the main removal process for trimethoprim and sulfamethoxazole, whereas the FQs' sorption to particulate matter is primarily responsible for their removal from surface water [44]. None of the compounds investigated is assumed to reside in water for a long time, due to their environmental properties. This does not imply that they cannot exert any negative effects on environmental organisms. For instance, FQs sorbed to sediment can affect organisms dwelling in that compartment.

It should be noted that a method blank containing tapwater from Borås was also analysed. This blank showed concentrations above LoQ for three of the FQs – 9 ng/L for norfloxacin, 11 ng/L for ofloxacin and 5 ng/L for ciprofloxacin. These concentrations are higher than any concentrations detected in both the outgoing water and the recipient water. The high concentrations are remarkable and too high to be ignored. The blank should, among other things, account for contamination of the samples. Though, it may not seem reasonable that the samples have been contaminated to this extent. The blank was analysed at the same time as samples from point E and F, which showed no detectable concentrations of the antibiotics, i.e. no contamination. In a subsequent analysis of tapwater from Umeå, the antibiotics could not be detected. Concentrating the different water samples showed that they differed much in colour – the sample from Borås seemed more contaminated. One may question if contamination exclusively from the laboratory method can be the only cause of contamination. One can also assume that the drinking water actually contained antibiotics. However, the inlet to the water purification plant in Borås is situated only a couple of hundred metres from where the recipient sample in Öresjö (point A, see map in Figure 6) was collected. This sample does, on the other hand, not show any detectable concentrations of the FQs. This means that if the drinking water is contaminated, it must have been during a very short period of time before its' inlet, or during the actual water purification process. There seems to be no simple argument explaining these results. However, the concentrations in the blank were used for correction of the concentrations in the other samples.

4.3.2. *Sediments samples*

The results from the analysis of the sediment samples could not be used to calculate concentrations of the antibiotics. Analyses of the calibration solutions could not be accomplished, since the peaks and their areas could not be identified. The peaks were not separated and the retention times appeared to be prolonged for each sample run, making the peaks appear outside their scanning period. The situation was found for both sediment samples and calibration solutions. The first two sample runs, with sample from Djupasjön, showed less delay in retention, but the prolongation then started to severely impair the results. The two first sample runs showed very small area ratios between analytes and internal standards, indicating that the concentrations of the antibiotics are probably below their individual LoQ. These results could be expected, since the FQs' environmental behaviour implies that the compounds should, in the recipient, be found in sediment rather than in the water phase. But since only very small amounts reach the recipient, it is not likely that the concentrations of these antibiotics in sediment will exceed the LoQ. The fairly low sorption of sulfamethoxazole to the solid phase, leads to the conclusion that this compound should rather be found in the water phase than in the sediment. This argument should also hold for trimethoprim, due to low sorption of the compound to sewage sludge [8].

Regarding the results from this experiment, it is assumed that the method used for extraction and analysis of the target antibiotics in sludge, is not suitable for sediment samples. The inapplicable results are probably due to various matrix effects. During this experiment it is assumed that the sample matrix has interacted with the column material, leading to longer retention of the analytes. It is established that samples with environmental matrices are complex and may be influenced by several interferences that affect the extraction and separation steps of the analysis [68]. In general, water samples are easier to work with than solid ones. When evaluating a method for determination of drugs in river sediments [11], the authors stressed that since the solvent extracts of the sediments contained a high level of natural matrix components, it was essential to include further clean-up steps to minimize chromatographic interferences and ion suppression during analysis. The method described in this reference includes alternative solutions for sample extraction, use of SPE cartridges and a different choice of solution for dissolving the extracts prior to analysis with LC. Additionally, the extracts are filtered before injection. Descriptions of other sample pre-treatment methods used also include alternative extraction procedures, extraction arrangements and clean-up routines [68].

It appears that analyses of antibiotics in river sediments are not frequently occurring, since only one literature-source concerning concentrations in this compartment could be found [40]. The analyses found usually concern marine sediments found under fish farms.

4.4. Environmental risk assessments

4.4.1. Predicted environmental concentrations (PEC) and measured environmental concentrations (MEC)

The calculated crude and refined PECs together with MEC are presented in Figure 9. It is clear that the crude PEC is not an accurate tool for predicting concentrations of these antibiotics in effluents from sewage treatment plants. The PEC_{crude} for ciprofloxacin for example, approaches 500 ng/L, whilst the actual, measured concentration of the substance is too low to be detected. Mind also that the predicted concentrations are calculated after dilution in the recipient, whilst MEC is measured before the effluent reaches the recipient.

The refined PEC must be seen as a more realistic approach to calculate concentrations since this term includes both metabolism in man and removal in the STPs. It is evident that these two mechanisms cannot be ignored, since some of the antibiotics examined are metabolized to a high extent – e.g. moxifloxacin is metabolized to 80% – and the FQs removal in the STP is also considerable. When including both metabolism and removal, the PECs for ciprofloxacin and norfloxacin are reduced more than 90%. The $PEC_{refined}$ gives a better picture of the actual concentration of the compounds, but is still several times higher than the measured concentrations. The calculation of PEC should preferably be further improved to become a more precise indicator of the presence and concentration of pharmaceuticals in the environment.

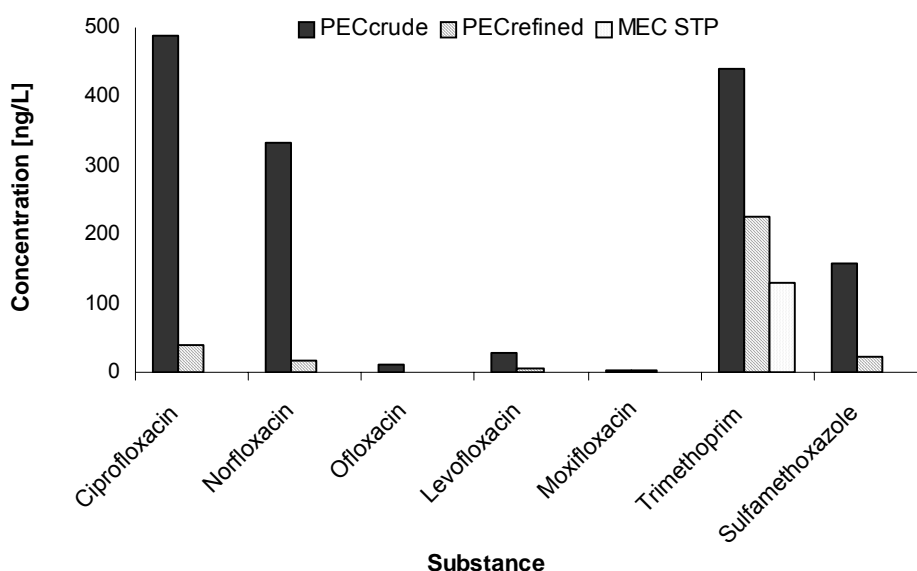


Figure 9. Comparison of PEC_{crude} , $PEC_{refined}$ and MEC for each antibiotic, expressed in nanograms per litre (ng/L).

The refined PECs for ofloxacin, levofloxacin and moxifloxacin are below the LoQ for ofloxacin for the method used. LoQ for moxifloxacin and levofloxacin are assumed to be equal to the LoQ for the other FQs. The refined PECs can therefore be accurate for these substances. This also holds for sulfamethoxazole, whose PEC_{refined} falls below its' LoQ of 80 ng/L. The predicted concentrations for levofloxacin and moxifloxacin are uncertain, since it is not known to which extent these are used in the region. The prescription of fluoroquinolones is not evenly distributed over the country, which means that these substances can be frequently used in the region, or not used at all.

4.4.2. Risk assessment: PEC/PNEC ratios and the utility and adequacy of ecotoxicological evaluations

According to the EMEA guideline, the first phase of the ERA should be followed by Phase II if the crude PEC exceeds 0,01 µg/L, which is the case for all substances except moxifloxacin. Phase II, level A of the ERA includes a calculation of the risk by dividing PEC by PNEC. The PNECs found in literature, and used for the calculation of PEC/PNEC, are presented in Table 8. Chronic toxicity test with effect concentrations were not available on different trophic levels for the target substances. There are some tests investigating the chronic effects of antibiotics on algae and bacteria, whilst most toxicity tests for higher organisms are performed under a short period of time. Antibiotics should not be on acute environmental concern since they are usually found at low concentrations and are discharged during a long period of time into the environment. Therefore, only chronic data are used in this ERA.

Table 8. Chronic toxicity data found for the target antibiotics in literature.

Substance	Lowest toxicity value found in literature	Species ^a	Reference
	[mg/L]		
Ciprofloxacin	EC ₅₀ = 18,7	<i>P. subcapitata</i>	[27]
Levofloxacin	EC ₅₀ = 7,4	<i>P. subcapitata</i>	[27]
Norfloxacin	NOEC = 4,01	<i>S. capricornutum</i>	[62]
Ofloxacin	EC ₅₀ = 1,44	<i>P. subcapitata</i>	[12]
Sulfamethoxazole	EC ₅₀ = 0,52	<i>P. subcapitata</i>	[12]
Trimethoprim	NOEC = 25,5	<i>S. capricornutum</i>	[62]

^a*P. Subcapitata* and *S. Capricornutum* are algae species.

When calculating risk using the MEC instead of PEC, none of the MEC/PNEC ratios exceed 1, since the antibiotics' concentrations detected in the final effluent waters are very low or not detected at all. Also when PEC_{crude} is used for risk calculation, all ratios are far below 1. The ERA can therefore be terminated. It is concluded that none of the antibiotics pose a risk to the environment in the current circumstances, according to the EMEA risk assessment guideline.

The ERAs present a very simplified version of the ecological impact of pharmaceuticals on the aquatic environment. The calculations do not take into account their presence in water and sediment, stability in the environment, presence of toxic metabolites etc. The primary obstacle for carrying out ERAs is the lack of toxicity data – the data found are usually from acute tests performed on a few species, often on algae and seldom on higher organisms. The lack of toxicity data may be due to the recent emergence of the issue of occurrence and impact of pharmaceuticals in the environment. Also, adequate methods for how to perform the toxicity test are believed to be insufficient [26].

The results derived from an ERA are based on several assumptions, e.g. the uncertainties emerged from a test is comprised to an uncertainty factor used to account for all uncertainties in a toxicity test. Moreover, a toxicity test-result for one or two species is used as a model for the whole ecosystem. What is more concerning is that in conventional ERAs, usually only one substance is considered in each assessment. Taking several compounds in account, preferably homogeneous compounds with similar structure and mode of action, when calculating the risk would give more credible results. This sort of ERA would account for potential additive toxicity for substances from the same class. The total concentration of the compounds in a class, for example the fluoroquinolones, should then be used together with the PNEC of the expected most potent compound when calculating the risk of this pharmaceutical mixture [16]. Concerning all the failings of today's risk assessment, one can ask whether they are a useful tool for predicting risk exerted by pharmaceuticals in the environment. However, they are the best methods for a risk forecast available at present.

The most serious shortcoming for ecotoxicological evaluations is that they are not adequate for the prediction of their effect in real nature. The short-term test with high concentrations does not reveal the actual effect of pharmaceuticals, since they are discharge during a long period of time at concentrations often too low to detect. As discussed above, most evaluations include testing of the effect from one single compound – a scenario unlikely to happen in nature. Obviously, effects deduced from authentic test need to be evaluated. However, a toxicity test may never reflect what really happens in nature, due to all the complex processes involved in the fate of a pharmaceutical compound. Though, it is evident that the negative effects of antibiotics cannot be reduced to the health effects caused by antibiotic resistance. The compounds' effect on primary producers can lead to adverse consequences for whole ecosystems, effects that are hard to predict today, due to lack of information.

The problem with antibiotics in the environment will most likely not be neglected, due to the threat of resistant bacteria on medical treatment of infectious diseases. Antibiotic resistance can principally be considered as a threat to human health, rather than an ecotoxicological problem. The impact of resistance on environmental organisms is not known, but the positive effects of antibiotics are of advantage mainly to humans and livestock, why the negative effects of resistance should in general affect these groups. Action to deal with resistance will in all likelihood be taken, since the cost of ignoring this problem, i.e. prolonged illness and increased mortality, will be too high. The problems related to antibiotic resistance should rather be derived from the use of these

substances within human and veterinary medicine, than from the discharge of the compounds in the environment. However, the impact of STP effluents containing antibiotic residues can expand in the future due to the increasing discharge of these substances into nature.

There is still not enough information to predict the pharmaceuticals' joint influence on environmental organisms. But since the amount of drugs discharged into the environment seems to increase in the close future, it is very likely that we will see more negative effects derived from these compounds. Hopefully, medicinal products will be given as much attention as other pollutants, such as dioxins and heavy metals, since it is now established that pharmaceutical compounds exist in various environmental compartments, and that they can exert negative effects on the organisms living there.

5. Conclusions

- Antibiotic compounds have earlier been detected in different environmental compartments, for example sewage sludge, surface water, soil, and sediment. The occurrence of antibiotics in nature is believed to lead to a selective pressure on resistant bacteria. Also, some tests have shown that antibiotics can affect the bacteria involved in the processes in STPs. These compounds are also reported to negatively affect non-target organisms. The effect on primary producers, such as algae, can cause effects on entire ecosystems.
- The concentrations of the fluoroquinolones analysed in this work ranged from 6 to 473 ng/L in raw sewage water, for norfloxacin and ciprofloxacin respectively. Trimethoprim was the only substance detected in the effluent water. Concentrations of the substances were all below LoQ in the environmental samples. The concentrations found in this experiment were normal compared to other investigations of sewage water.
- A comparison of PEC and MEC, show that PEC is several times enhanced compared to the measured values, even when metabolism and removal in STPs are taken into account. Performing the initial steps of the EMEA risk assessment, showed that none of the substances exert an environmental risk. However, performing risk assessments can be difficult due to lack of data, which lead to uncertain results.

6. Future remarks

Due to the lack of results from the analysis of antibiotics in the sediments, another method for preparation of the samples should be employed. Today, methods for analysis of most human and veterinary medicinal products in sediments are not available. There are a few methods evaluated, allowing the determination of e.g. sulfamethoxazole and trimethoprim residues in sediments down to the lower ng/g range [11, 68]. It is plausible that one of the methods described in these references should end in useful results for the sediments analysis.

Since very few investigations of river and marine sediments have been performed, new analysis of sediments close to sewage discharges should be accomplished. These can reveal whether antibiotics accumulate in sediments, which could lead to negative ecological effects.

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Appendix 1. (Sampling Points)

Water samples

- A. Öresjö – close to the inlet of the water purification plant of Borås. Situated upstream from Borås population centre.
- B. ~ 150 m downstream from Gässlösa STP.
- C. Jössabron – located ~ 500 m downstream from Gässlösa STP.
- D. Osdalbron – situated ~ 1 000 m downstream from Gässlösa STP.
- E. Djupasjön – closer to the shore.
- F. Djupasjön – closer to the main stream of Viskan.
- G. Ginkalunda
- H. Kinnaström – located upstream from Kinna population centre.
- I. ~ 200 m downstream from Skene STP.
- J. Main road 41 – bridge located ~ 750 m downstream Skene STP.
- K. Viskadal – bridge located ~ 2 400 m downstream Skene STP.
- L. Åsbro – point before Viskan's outlet in Kattegatt. Åsbro is an important sampling point for the Viskan Vattenvårdförbund, since the point represents the amount of substances transported from Viskan into the ocean.

Sediment samples

- 1. Öresjö
- 2. Jössabron
- 3. Djupasjön
- 4. Åsbro

**Analyser av östrogena ämnen i
Gässlösa reningsverk och Skene reningsverk
samt i recipienten Viskan**

**Rapport av Karin Björklund
2005**

Sammanfattning

Förekomsten av de östrogena substanserna östron, östradiol, östriol och etinylöstradiol, samt progesteronet norethindron har undersökts i in- och utgående avloppsvatten i Gässlösa reningsverk (Borås) och Skene reningsverk (Mark), samt i recipienten för avloppsvattnet, Viskan. Östrogena ämnen är hormoner som ansvarar för utvecklingen av kvinnliga könsattribut, såsom livmoder och äggstockar, samt regleringen av den reproduktiva cykeln. De östrogena föreningarna har påvisats i många vattendrag där avloppsvatten släpps ut, dessutom har man sett att de kan utöva negativa effekter på fisk och andra organismer som lever i dessa vatten. En vanlig effekt är ökad syntes av vitellogenin (VTG) hos fiskhanar och icke könsmogna honor – VTG är ett ägguleprotein som normalt bara produceras av könsmogna fiskhonor. För fisk och andra vattenlevande organismer kan denna effekt leda till infertilitet och rubbade könskvoter. Fiskar i engelska vattendrag har också uppvisat ”intersex” – simultan förekomst av både hanliga och honliga könsegenskaper – vilket kan visa sig som förekomst av äggstockar hos hanar och outvecklade ägg i testiklarna.

Hormoner bildas naturligt i kroppen, men kan också användas i medicinsk behandling av bl a klimakteriebesvär och som preventivmedel. Substanserna kan antingen förbli aktiva eller ombildas i kroppen till inaktiva föreningar, innan de följer med urin och avföring och transporteras med avloppsvatten till reningsverken. I reningsverken kan föreningarna ombildas till aktiva substanser igen, eller brytas ned och inaktiveras m h a olika kemiska och biologiska metoder. De processer i reningsverken som ger högst avlägsnandegrad för östrogen, är aktivt slam och kväverening. Det finns dock ingen metod som generellt alltid ger komplett nedbrytning av substanserna och dessutom kan ett och samma reningsverk uppvisa stor variation på nedbrytningen beroende på tidpunkt för provtagning.

De kemiska analyserna utförda i denna undersökning visade att koncentrationerna av etinylöstradiol och norethindron understeg detektionsgränsen i alla prover. Däremot påvisade man östron, östradiol och östriol i alla inkommande avloppsvattenprover och de flesta utgående prover, med undantag för östradiol och östriol som i Skene reningsverk låg under detektionsnivån. Östron detekterades också i varierande halt i recipientproverna från Viskan. Över lag ser man att reningseffekten i Skene reningsverk är högre än den i Gässlösa, som t o m visar förhöjda halter östradiol i utgående jämfört med inkommande vatten. Liknande resultat har även dokumenterats i en annan undersökning, vilket tyder på att Gässlösa inte klarar av att rena vattnet från östrogen lika bra som Skene. De båda verken begagnar sig av liknande reningssteg, varför reningseffekten också borde sammanfalla. Den enda skillnaden som kan tänkas ge bättre avlägsnande av substanserna i Skene, är att avloppsvattnet där tillbringar längre tid i de biologiska reningsstegen, vilket kan leda till högre nedbrytning av hormonerna.

Två undersökningar, utförda av IVL respektive Göteborgs Universitet, där man i burar satt ut fiskar i Viskan, uppvisar skilda resultat – i den ena undersökningen påvisas ingen förhöjd VTG-halt hos fiskarna, medan man i det andra testet ser onaturligt höga VTG-koncentrationer hos vissa fiskar. Där man sett förändringar i VTG-halten, drar författarna slutsatsen att påverkan kommer från reningsverkens utsläpp, man kan dock inte avgöra om det beror av historiska utsläpp som ansamlats i åsedimenten, eller om det beror av pågående utsläpp. Det är, med endast dessa resultat som grund, svårt att dra en slutsats huruvida östrogenkoncentrationerna i Viskan är höga nog för att ge negativa effekter hos de fiskar som lever däri.

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1. Introduktion

Naturliga hormoner och dess nedbrytningsprodukter har alltid förekommit i miljön [1]. Under de senaste årtiondena har användningen av naturliga och syntetiska östrogen och progesteroner inom både human- och veterinärmedicin dock ökat stadigt. Östrogen används idag i bl a preventivmedel och som behandling av problem vid och efter menopausen. Den ökande användningen av dessa substanser speglas nu av allt högre hormonkoncentrationer i olika naturliga miljöer. De hormoner som utsöndras av människor når med avloppsvattnet reningsverk, där elimination av substanserna sker till olika grad [2]. Under 1980-talet uppmärksammades problemen med förekomsten av hormonstörande ämnen i kommunalt avloppsvatten i Storbritannien [3]. De förhöjda koncentrationerna av dessa substanser nedströms reningsverken, kunde man snart koppla samman med missbildningar hos fiskar i dessa vattendrag. Många undersökningar har efter detta bekräftat att både naturliga och syntetiska östrogen kan transporteras med det utgående avloppsvattnet och spridas i recipienten, där de kan utöva olika negativa effekter på vattenlevande organismer [2]. Man har bevis för att kvinnliga könshormoner kan leda till feminisering, intersex, d v s en blandning av honliga och hanliga drag, samt fortplantningsstörningar hos fiskar [4]. Andra studier har visat att också fåglar, reptiler och däggdjur genomgår förändringar i sitt reproduktiva system när de utsätts för förhöjda koncentrationer östrogen. Alla ryggradsdjur utsöndrar själva hormoner och påverkas därför också av dessa substanser, även om de är kroppsfrämmande [5]. Eftersom alla östrogen har liknande kemisk struktur, kan samma substans påverka hormonella system hos många olika arter [6]. Små modifieringar i den hormonella funktionen kan leda till bl a förändringar i tillväxt, utveckling eller reproduktion hos den utsatta organismen och dessa förändringar kan uttryckas senare i livscykeln eller till och med i kommande generationer [7].

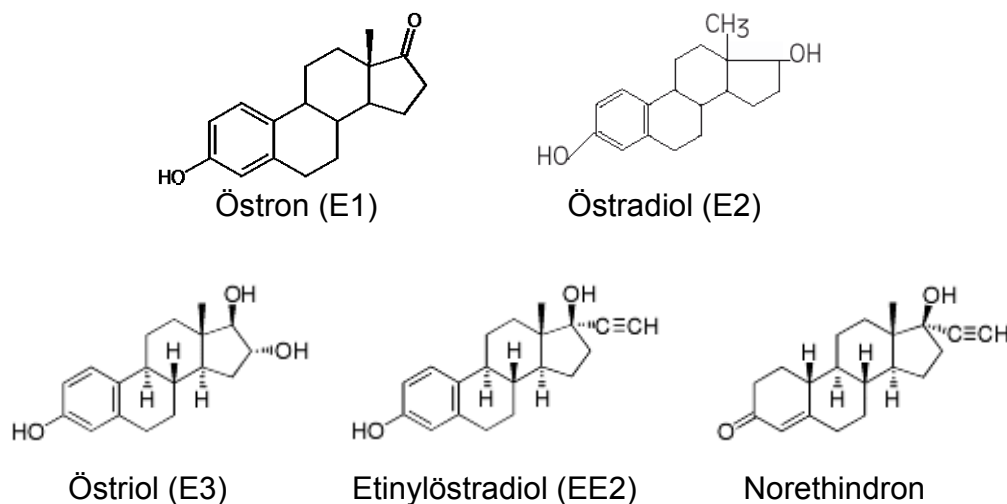
Hittills har man undersökt ämnen med östrogen effekt – motsvarande effekter från de androgena substanserna, d v s manliga hormoner, är ännu mycket litet kända [8]. Utöver de naturliga och syntetiska hormonerna, finns det miljöfrämmande kemiska substanser som av organismer uppfattas som östrogenliknande och därför ger samma effekter som hormonerna [9, 10]. Bland dessa ämnen hör t ex olika fenoler, PCB, DDT och många bekämpningsmedel [11]. Denna undersökning innefattar dock endast analyser av naturliga och syntetiska hormoner och rapporten behandlar därför bara dessa ämnen.

Till och med vid mycket låga koncentrationer – ≥ 1 ng/l – ger östrogena substanser effekter i form av hormonella störningar hos vattenlevande organismer [9, 12, 13]. På grund av deras höga östrogena verkan, har man, i de flesta vetenskapliga undersökningar, fokuserat på att utvärdera förekomsten och effekten av naturliga östrogen; östron (även kallat E1), östradiol (E2) och östriol (E3), samt det mest använda, syntetiska östrogenet etinylöstradiol (EE2). I denna undersökning har man dessutom valt att analysera förekomsten av norethindron – ett progesteron som förekommer i preventivmedel [14]. Förekomsten av E1, E2, E3, EE2 och norethindron har undersökts i både det orenade avloppsvattnet och det utgående, reade vattnet från Gässlösa reningsverk i Borås, samt Skene reningsverk i Marks kommun, Västra Götaland. Man har även undersökt halten av de ovan nämnda substanserna vid fem provtagningspunkter i avloppsverkens recipient, Viskan.

2. Bakgrund

2.1 Hormonernas funktion och användning

Det hormonella systemet är det som kontrollerar många av en organisms funktioner [6]. Hormoner kan ses som kemiska budbärare som interagerar med specifika receptorer eller celler och kan slå på, eller av, olika funktioner i cellen och därmed förändra dess fysiologiska status. Könshormoner, vilka påverkar reproduktionen, tillhör gruppen steroidhormon, som hos ryggradsdjur produceras i testiklar och äggstockar [15]. Alla steroidhormoner härstammar från kolesterol och har därför liknande kemisk struktur, se Figur 1. Reproduktionshormonerna delas ofta upp i tre grupper; androgener, östrogener och progesteroner. Både androgener och östrogener är viktiga för båda könen med avseende på tillväxt, utveckling och regleringen av sexuella och reproduktiva beteenden. Androgener dominerar dock i manliga individer, där hormonet ansvarar för utvecklingen av manliga attribut, såsom testiklar och tuppens kam. Östrogener, som finns i högre koncentration hos kvinnliga individer, stimulerar utvecklingen av exempelvis livmoder och äggstockar samt regleringen av den reproduktiva cykeln. Progesteroner är ansvariga för att förbereda kroppen för graviditet, och bibehålla graviditeten genom att gynna utvecklingen av det befruktade ägget och tillväxten av livmodershinnan.



Figur 1. Kemisk struktur för östron, östradiol, östriol, etinylöstradiol samt norethindron [14].

Preventivmedel innehåller oftast en kombination av syntetiskt framkallade östrogener och progesteron med samma effekt som de naturliga [14]. Syntetiskt östrogen, i form av etinylöstradiol, är identiskt i alla tabletter, medan progesteronet, bl a norethindron (även kallat noretisteron), varierar mellan olika p-piller. Östradiol, den viktigaste, naturligt förekommande östrogenerna, är också den i Sverige mest använda substansen för att behandla vanliga klimakteriebesvär. Östron och östradiol utgör medelpotenta naturliga hormoner, medan östriol anses vara ett ”svagt” östrogen och etinylöstradiol ett mycket potent hormon [7, 10, 14, 16]. Av medicinska skäl har de konstgjorda hormonerna framställts så att de är mer stabila än de naturliga, vilket leder till att de oftast har längre verkningsstid och högre biologisk aktivitet än de naturliga. Därför kan lägre doser av ett syntetiskt hormon ge samma effekt som en högre dos av ett kroppseget hormon.

Tabell 1. Sammanfattande information om de analyserade hormonerna för att underlätta fortsatt läsning.

Substans	Alternativ beteckning	Ursprung	Egenskaper
Östron	E1	naturligt östrogen	medelpotent
Östradiol	E2	naturligt östrogen	medelpotent
Östriol	E2	naturligt östrogen	svagt
Etinylöstradiol	EE2	syntetiskt östrogen	potent, stabilt
Norethindron	noretisteron	syntetiskt progesteron	ingen uppgift

2.2. Kroppslig nedbrytning och utsöndring av östrogena ämnen

Hormonella substanser bryts till olika grad ned i kroppen och utsöndras därefter med urin och avföring [12, 14]. Innan de utsöndras genomgår substanserna oftast någon transformation – de oxideras, hydroxyleras eller metyleras innan de tills sist konjugeras med glukuronsyra eller sulfat. Utsöndringen av metaboliter sker i huvudsak via njurarna i form av glukuronider och sulfater. Transformationen medför oftast att östrogenerna avsöndras i en biologiskt inaktiv form, som inte kan påverka hormonella system. Naturliga hormon metaboliserar snabbare i kroppen och utsöndras i högre grad som inaktiva föreningar än de syntetiska varianterna, vilka inte tas upp lika bra av kroppen [1]. Östradiol oxideras till östron, som i sin tur kan omvandlas till östriol, men andra metaboliter kan också bildas vid nedbrytningen av östradiol [12, 14]. Både norethindron och etinylöstradiol utsöndras till stor del som glukuronidderivat via urin – norethindron huvudsakligen metaboliserat och etinylöstradiol i såväl oförändrad som metaboliserad form.

Hur stor mängd hormoner som utsöndras naturligt från kroppen beror bl a på kön och i vilken fas i livet man befinner sig i [12]. Under graviditeten utsöndrar kvinnor mycket högre mängder än vid andra perioder. Män producerar nästan lika mycket östrogen som äldre kvinnor och yngre flickor [10]. Kvinnor som använder sig av preventivmedel utsöndrar i regel runt 35 µg etinylöstradiol per dag (se Tabell 1).

Tabell 2. Daglig utsöndring [µg] av östron (E1), östradiol (E2), östriol (E3) samt östradiol (EE2) hos människor [12].

	E1	E2	E3	EE2
Män	3,9	1,6	1,5	-
Menstruerande kvinnor	8	3,5	4,8	-
Kvinnor i menopaus	4	2,3	1	-
Gravida kvinnor	600	259	6000	-
Kvinnor som tar preventivmedel	-	-	-	35

Efter utsöndring i urin och avföring, transporteras hormonerna och dess metaboliter med avloppsvatten till de kommunala reningsverken, alternativt enskilda avlopp [7, 9, 12]. Vad som händer med de hormonella substanserna i enskilda avlopp är oklart, eftersom inga sådana undersökningar genomförts. Avloppsvatten från hushåll, arbetsplatser etc är den dominerande spridningsvägen för hormoner från människokroppen ut i miljön. Dagens reningsverk har en reningsprocess som är effektiv för att avlägsna bl a fosfor och lättnedbrytbart organiskt

material. Andra typer av föroreningar, främst svårnedbrytbara och vattenlösliga substanser, passerar oftast verken oförändrade och går därmed ut med behandlat avloppsvatten till recipienten, oftast en å eller älv.

2. 3. Vad sker med hormonerna när det nått reningsverket?

Trots att majoriteten av de östrogener som utsöndras av människor, återfinns i dess biologiskt inaktiva, konjugerade form, har man detekterat höga halter av ”fria” hormoner i reningsverk och dess recipienter, vilket tyder på att östrogenmetaboliter kan återombildas till dess aktiva form i avloppsvattnet, någonstans mellan toaletten och reningsverkens utlopp [4, 12]. Aktiva substanser kan, om de transporteras ut med det utgående vattnet, påverka hormonbalansen hos organismer i recipienten [10].

Undersökningar har visat att koncentrationen av både östron och östradiol ökar efter de första reningsstegen i ett avloppswerk, vilket tyder på att konjugaten klyvs och hormonerna ombildas till dess aktiva form [13]. Östradiol elimineras dock ofta eftersom i reningsverken, bl a kan den transformeras till östron. Även andra metaboliter konjugerade med glukuronsyra har visat sig kunna omvandlas till östron under transporten genom reningsverk [4], vilket leder till en ökande koncentrationen av detta ämne i de följande reningsstegen. Det finns också det som tyder på att bl a norethindron kan ombildas till etinylöstradiol [16]. Dessutom kan lynestrenol, som används i vissa preventivmedel, metaboliseras till norethindron, som i sin tur omvandlas till etinylöstradiol. I orenat avloppsvatten och i reningsverk förekommer höga halter mikroorganismer, som kan klyva de inaktiva konjugerade östrogenerna, så att den aktiva formen av substansen återbildas [4, 12]. Bland annat har det visat sig att *E.coli*, rikligt förekommande i avföring, kan syntetisera ett enzym som antas kunna inducera klyvningen av konjugaten. Detta kan också förklara varför 85-90% av de hormon som utsöndras med feces är i dess fria form [4].

Beroende på vilken metod som används för att rena avloppsvatten, kan östrogenerna avlägsnas till olika grad från vattnet [10]. Det finns inga klara besked om vilken sorts reningsteknik som genomgående ger högst avlägsnande av hormonsubstanser. Man har dock kunnat se vissa mönster i reningseffekten – bl a har biologisk rening gett en elimination av E2 på mellan 75-98% i kanadensiska reningsverk [7]. Nedbrytningen av E1 är mer komplicerad och har visat sig variera från näst intill fullständig nedbrytning till en koncentrationsökning av hormonet mellan inlopp och utlopp. Samma resultat, d v s hög elimination av E2 och varierande nedbrytning eller förhöjda koncentrationer av E1, har man fått vid undersökning av italienska reningsverk [4]. Överlag ser man att EE2 är mer persistent i avloppsvattnet och -slammet [16].

Tabell 3. Uppmätta koncentrationer av östron (E1), östradiol (E2), östriol (E3), samt östradiol (EE2) i inkommande/utgående avloppsvatten [ng/l] samt verkens reningseffekt [%].

Provtagningsplats	Reningssteg	E1 in/ut [ng/l]	E2 in/ut [ng/l]	E3 in/ut [ng/l]	EE2 in/ut [ng/l]	Reningseffekt E1/E2/E3/EE2	Referens
Gässlösa	Trickl ^a /AS ^b , kväve ^c		e.a. ^d /5,0-7,7 ^e				[9, 10, 17, 18]
Skene	Trickl/AS, kväve		e.a./1,9-3,8 ^e				[9, 10, 17, 18]
Sverige	8 olika ^f		1,1-29,6/<0,1-15 ^e			-257 - ≥99	[10]
Kävlinge	AS, kväve	49/>0,3	e.a./>0,9	e.a.	e.a.	>99/e.b. ^g	[19]
Italien	Inte känt	52/19	12/1,4	80/3	e.a.	63,5/88,3/96,3	[4]
Storbritannien	Inte känt	e.a./1-80	e.a./1-50	e.a.	e.a./0-7,0		[17, 18]
Nederländerna	Inte känt	11-140/<0,4-47	<LoQ ^h -48/<0,1-5	e.a.	>0,2-8,8/<0,2-7,5		[12]
Tyskland	Inte känt	e.a./70	e.a./3	e.a.	e.a./15		[17, 18]
Kanada	AS, kväve	79/2	17/<1	e.a.	e.a.	97,8/>98	[7]
Kanada	AS, kväve	39,7/13	7/1,2	e.a.	e.a.	66,7/82,9	[7]
Kanada	Trickl	63/103	13/15,5	e.a.	e.a.	-62,4/-18,5	[7]

^a Tricklingfilter

^b Aktivt slam

^c Steg för kvävereduktion

^d Ej analyserat

^e Östradiolekvivalenter. Biologiskt test av hormonkoncentrationen. Ger ingen absolut koncentration av de olika hormonella substanserna, utan ett värde uttryckt i östradiolekvivalenter, se vidare i texten.

^f Ingen biologisk rening, AS, AS + biosorption, tricklingfilter, biorotor, tricklingfilter/AS, AS + kvävereduktion, tricklingfilter/AS + kvävereduktion

^g Ej beräknat

^h Under LOQ = limit of quantification, d v s under kvantifieringsnivån

En undersökning av svenska avloppsreningsverk bekräftar att biologisk rening är viktig för att minska halterna av östrogena ämnen, vilket tros ha ett samband med förekomsten av mikroorganismer, som kan bryta ned och reducera halterna av östrogener [17]. I den svenska undersökningen hade de flesta verk lägre halter östrogener i det utgående än i det inkommande vattnet, men man såg också en förhöjd koncentration av vissa substanser i det renade vattnet. De verk som uppvisade högst reduktion av hormon innehöll steg för kväverening, vilket man även sett i den kanadensiska undersökningen [7]. Den förhöjda reduktionen av hormoner i dessa verk kan ske antingen i det anoxiska steget, eller, vilket är mer troligt, bero av en ökad uppehållstid för avloppsvattnet i det biologiska steget. Reningsverk med långa uppehållstider i det biologiska steget har över lag visat sig ha ökad reduktion av hormonsubstanser [7, 9, 17]. De svenska verk som visade sig ha lika höga halter i det utgående som i det inkommande vattnet, använde sig av direktsedimentering utan biologisk rening [9, 17, 18]. System som bara innefattar kemisk fällning med Al eller Fe gav inte hellre någon effektiv reduktion av hormon. Man har dock sett att upprepade fällningssteg leder till högre reduktion av ämnen med östrogena effekter [10].

När man undersökte effektiviteten hos olika biologiska reningsmetoder, såg man att tricklingfilter inte ger en tillfredsställande reduktion av östrogen – endast ca 1/3 av ämnena avlägsnas [9, 10, 17]. Dessa resultat överensstämmer med en tysk undersökning, där användningen av aktivt slam-processer gav genomgående lägre halter än tricklingfilter [18]. En Brasiliansk undersökning [18] publicerade en avlägsningsgrad på mellan 67-83 % för E1, 92-99,9 % för E2, samt mellan 64-78 % för EE2 vid aktivt slam-rening. En kanadensisk undersökning demonstrerar tydligt att verk med liknande reningsprocesser uppvisar mycket varierande reningseffekt av östrogener [7]. I verk med aktivt slam-process samt kväverening har reduktionen av E1 varit så hög som 98,8 %, men halten av samma ämne har också ökat med närmare 50 % i ett annat verk (se även Tabell 3). Över lag har man dock sett att reningsverk med kvävereduktion ger ett högre avlägsnande av både E1 och E2.

Många forskare anser att reduktionen av östrogener och östrogen effekt i kommunala reningsverk är mycket komplex och fortfarande inte väl utredd eller förstådd. Multipla undersökningar av samma verk visar också att reduktionen av dessa ämnen kan variera högst betydligt, beroende på när man tagit proven [10]. Samma verk har uppvisat variationer på retention av östrogener på ett tiotal procentenheter.

Eftersom östrogener är hydrofoba organiska föreningar med låg flyktighet, kan man förvänta sig att de absorberas och återfinns i slam från reningsverk [12]. I både aktivt och stabiliserat slam har man kunnat detektera relativt höga halter östrogener – 37 ng/g E1, 49 ng/g E2, samt 17 ng/g EE2, vilket tyder på att östrogener kan förbli oförändrade under slambehandling [1].

2. 4. Förekomst av hormoner i olika miljöer

Avloppsvatten är största källan för hormoner i naturen men på god andra plats kommer spridningen från djur [1]. Östrogener finns naturligt i djur, men kan också användas i medicinskt syfte för att uppnå olika resultat [5]. Steroider används vanligen för att kontrollera brunst, åtgärda reproduktiva problem och inducera abort [12]. Olika arter utsöndrar olika typer och mängder av östrogener [2] och precis som för människor varierar utsöndringen av hormon beroende på om djuret är dräktigt och med vilket skede av graviditeten djuret befinner sig i [5]. Mjölkkor beräknas utsöndra ca 838 µg E1 och 384 µg E2 per ko och dag. Grisar kan utsöndra upp till 1400 µg E1/gris/dag om de är dräktiga. En beräkning gjord av

forskare i Storbritannien visar att landets 157,5 miljoner husdjur förväntas utsöndra nästan 790 kg östradiolekvivalenter per år jämfört med den mänskliga utsöndringen på ca 220 kg, dvs nästan en fjärdedel så stor. I Sverige finns ca 10,5 miljoner husdjur och 9 miljoner människor, jämför med Storbritanniens 59 miljoner, vilket innebär att utsöndringen av hormoner från djuren inte har lika stor inverkan på den totala spridningen i Sverige [20, 21].

Huvuddelen av djurens hormoner återfinns i urin och avföring [1]. När denna gödsel sprids på jordbruksmark kan hormonerna och dess metaboliter, som ett resultat av urlakning eller avrinning vid nederbörd, nå grundvatten respektive ytvatten och där påverka levande organismer. Östradiol har detekterats i lakvatten från gödslad mark och även i grundvatten [12]. Man har också sett att spridning av hormoner och andra miljöstörande ämnen kan ske vid användning av avloppsslam som gödningsmedel på jordbruksmark [1]. Det är dock inte noga utrett huruvida östrogener snabbt bryts ned, adsorberas till jordpartiklar eller transporteras till omgivande miljö. Nedbrytningen av steroider är en komplex mikrobiell process som bl a beror av dess koncentration, tillgång på näring och förekomsten av stabila mikrobiella kolonier.

Flera undersökningar har bekräftat förekomsten av hormonella ämnen i ytvatten, där de kunnat detekteras i både vattenfasen och sedimenten. En sammanfattning av några resultat finns att se i Tabell 4. Undersökningar av förekomsten av norethindron är mycket få. Analys av detta ämne har inte prioriterats och metoder för att kunna analysera ämnet är fortfarande under utveckling [22, 23]. En tidigare undersökning av östrogenerna ämnen i Viskan demonstrerar varierande östrogenicitet längs ån [9, 10, 17]. Något förhöjda värden – mellan 4,1 och 8,2 ng/l östradiolekvivalenter (EEQ) – uppmättes mellan Gässlösa och Skene reningsverk, vilket tyder på att östrogeniciteten främst beror av utsläpp från reningsverken. I den ovan nämnda undersökningen användes biotest, vilket endast ger ett sammanfattat mått på koncentration av de hormonella substanserna, uttryckt i EEQ [9, 10, 17]. I testet användes jästceller med modifierade gener, i detta fall en gen för den humana östrogenreceptorn. Genen uttrycks när den utsätts för östrogen och en reporter-gen (här ett enzym) visar sedan detta uttryck genom att ändra färg. Beroende på hur mycket färgen uttrycks, kan man, med hjälp av kalibreringslösningar, beräkna koncentrationen uttryckt i östradiolekvivalenter. Skillnaden mellan detta biotest och kemisk analys, är att man i en kemisk analys får fram den absoluta koncentrationen för de enskilda substanserna som man valt att analysera och kan på så sätt se om de återfinns i olika höga koncentrationer. Koncentrationen av östrogenerna substanser i recipienten, i tidigare nämnd undersökning [10], avslöjade vissa avvikelser från den koncentrationsgradient som man förväntat sig längs ån, vilket kan bero på inverkan från andra, mindre reningsverk.

Steroiderna har mer eller mindre lång halveringstid ($t_{1/2}$) i vatten, beroende på hur mottagliga de är för nedbrytning [24]. Naturliga hormoner kan halveras inom 2-6 dagar, medan $t_{1/2}$ för etinylöstradiol var 46 dagar i samma försök. Larsson har sett att kvoten mellan EE2 och naturliga östrogener i vatten är högre än den teoretiska kvoten baserad på mänskliga utsöndringshalter av naturliga och syntetiska hormoner [16], vilket bekräftar tesen om att EE2 bryts ned långsammare än de naturliga substanserna.

Tabell 4. Uppmätta koncentrationer av östron (E1), östradiol (E2), östriol (E3), östradiol (EE2) samt norethindron (Nor) i olika naturliga vattenmiljöer.

Provtagningsplats	E1	E2	E3	EE2	Nor	Referens
Innanhav (Stockholm, Sverige)	0,2-2,7 ng/l	e.d. ¹	e.a. ²	e.d.-3,6 ng/l	e.a.	[25]
Div. ytvatten (Nederländerna)	<0,1-7,2 ng/l	<0,3-5,5 ng/l	e.a.	<0,1-4,3 ng/l	e.a.	[12, 26]
Div. vattendrag (USA)	27 ³ (112 ⁴) ng/l	160 (200) ng/l	19 (51) ng/l	71 (831) ng/l	48 (872) ng/l	[27]
Flodvatten (Italien)	1,5 ng/l	0,11 ng/l	0,33 ng/l	0,04 ng/l	e.a.	[12]
Flodvatten (Israel)	1,2-9,4 ng/l	1,2-9,4 ng/l	0,7->40 ng/l	0,2->40 ng/l	e.a.	[24]
Flod (Japan)	e.a.	2,1	e.a.	e.a.	e.a.	[12]
Flodvatten (UK)	e.d.-7,1 ng/l	e.d.-25 ng/l	e.a.	e.d.-0,038 ng/l	e.a.	[6]
Flodvatten (Danmark)	e.a.	e.a.	e.a.	0,2-15 ng/l	< 1 µg/l	[28]
Flodvatten (Spanien)	2,4-4,4 ng/l	2,4-3,0 ng/l	e.a.	>LoQ ⁵	e.a.	[13]
Flodvatten (Tyskland)	0,1-4,1 ng/l	0,15-3,6 ng/l	e.a.	0,1-5,1 ng/l	e.a.	[12]
Flodsediment (UK)	>0,04-0,388 ng/g	e.d.	e.d.	e.a.	e.a.	[1]
Flodsediment (Spanien)	11,9 ng/g	e.a.	e.a.	22,8 ng/g	"ofta förekommande" ⁶	[1]

¹ Ej detekterat.

² Ej analyserat.

³ Median-värde.

⁴ Maximalt uppmätt värde.

⁵ Under LoQ = limit of quantification, d v s under kvantifieringsnivån

⁶ Ofta förekommande i analys av sediment, men ingen koncentration nämnd.

En spansk forskargrupp har undersökt huruvida östrogener adsorberas till organiskt material och sett att dessa ämnen uppvisar en generell tendens att ackumuleras i sediment, p g a deras fysikaliska och kemiska egenskaper [1]. Flera europeiska undersökningar har i flodsediment kunna detektera flertalet östrogener (E1, E2, E3, EE2) och progesteroner (bl a norethindron) i halterna 0,04-22,8 ng/g (se Tabell 3). Östrogener kan uppehålla sig i sediment under lång tid, transporteras till andra, opåverkade områden och så småningom frigörs till det omgivande vattnet [1, 3]. Organismer som uppehåller sig i sedimenten kan sedan exponeras för de hormon som utsöndrats från människor. Man har till och med kunnat se hormonella störningar hos organismer som exponerats för kontaminerat sediment.

2. 5. Effekter av östrogena ämnen på levande organismer

Det är ingen tillfällighet att nästan alla väldokumenterade exempel på hormonella störningar i vilda djur har upptäckts antingen hos djur som lever i vatten, t ex alligatorer i USA och fisk i Storbritannien, eller djur som lever av dessa vattendjur, såsom fiskätande fåglar [29]. Det är befogat att tro att dessa organismer utsätts för högst koncentrationer av östrogena substanser och därför också är de som påverkas mest. Östrogener är kända för att utöva många olika effekter på fiskar [9]. Till dessa hör kontroll av vitellogeninsyntes, produktion av äggskalsprotein, förändring av könskörtlar, utveckling av sekundära könsegenskaper, syntes av östrogen-receptorer, feromonisk kommunikation och kalcium-homeostas. Östrogener i naturen har därför potential att störa många olika normala fysiologiska funktioner. Det är nu över 20 år sedan man upptäckte hermafrodita fiskar intill engelska reningsverk [10]. När man satte ut burar med fisk i vattnet nedströms verken, ökade syntesen av vitellogenin, som fungerar som en mycket känslig och specifik biomarkör för exponering av östrogena ämnen. Vitellogenin (VTG) är ett ägguleprotein som normalt bara produceras av könsmogna fiskhonor. När fiskhanar och icke könsmogna honliga fiskar utsätts för östrogena substanser induceras VTG-produktionen och höga halter kan uppmätas i blodet [16, 29]. För fisk och andra vattenlevande organismer kan detta leda till infertilitet och rubbade könskvoter [9, 29]. En brittisk undersökning där man fångat vild forell både uppströms och nedströms flera reningsverk uppvisade under en histologisk granskning av könskörtlarna att hanarna var ”intersex”, vilket definieras som en simultan förekomst av både hanliga och honliga könsegenskaper [9, 29]. Detta kan vara förekomst av outvecklade ägg i testiklarna, äggstockar och även saknad av sädesledare [30]. Förekomsten av intersexualitet varierade mellan 4 till 100 % av hanarna i de åtta undersökta vattendragen [9, 29]. Även fiskar som lever i marina miljöer har uppvisat förhöjda nivåer av vitellogenin och ökad förekomst av könsorgan som är ett mellanting mellan honliga och hanliga drag [9]. I de områden där dessa effekter påvisats, sker utsläpp av kommunalt avloppsvatten. Intersex har detekterats i vilda bestånd av bl a mört, flundra, tånglake, braxen och abborre i, förutom Storbritannien, Frankrike, Spanien, Holland, Tyskland och Danmark [30]. Exponeringen av östrogena substanser som inducerar syntes av VTG anses också vara metaboliskt stressande för fiskar av båda könen [10]. Denna stress kan leda till skador på njurar och lever samt att kalk frisätts från skelett och fjäll. Än vet man inte vilken effekt intersex och ökade koncentrationer av VTG har på hela populationer [26, 29]. Man vet inte om fiskar med intersex kan producera könsceller, om könscellerna i så fall skulle kunna frisättas och om de skulle vara livsdugliga. Nyligen presenterade resultat som visar på att den reproduktiva förmågan hos fisk med intersex kan vara reducerad. Denna effekt kan ha en negativ inverkan på reproduktionen och överlevnaden hos dessa populationer [26]. Det är också troligt att östrogener kan påverka även fysiologiska processer, vilket är fallet hos däggdjur, och inte bara ge reproduktiva effekter hos fiskarna [29].

Det finns också flera exempel på svenska undersökningar där man hållit fiskar i bur i vatten nedströms reningsverk. Larsson et al visade att halten östrogena substanser var 10^4 - 10^6 gånger högre i fiskens galla än i det omgivande vattnet, vilket tyder på att substanserna biomagnifieras i fisken [16]. IVL:s undersökning [10] visade att endast en av 175 undersökta regnbågar, fördelade på 23 olika kontrolllokaler, hade en vitellogeninhalt i blodet överstigande detektionsgränsen på 400 ng/ml. Efter tre veckors exponering kunde man alltså inte påvisa någon effekt av östrogena ämnen. Dessa resultat överensstämmer dåligt med många av de resultat man sett i engelska undersökningar. Troligtvis har man i de svenska recipienterna större utspädning av avloppsvattnet, vilket gör att fiskarna exponeras för lägre hormonkoncentrationer. När man i IVL:s undersökning exponerade fisken för utspätt, behandlat kommunalt avloppsvatten, erhöll man däremot förhöjda VTG-halter. Denna utredning innefattade vatten från Gässlösa och Skene reningsverk, som alltså uppvisade östrogena effekter i form av ökade halter av VTG. Detta gällde dock utspätt avloppsvatten och effekterna var jämförelsevis låga. Motsägande resultat har avslöjats när Zoologiska institutionen vid Göteborgs Universitet undersökte vitellogeninhalten i burutsatt, juvenil regnbåge [31]. Fiskarna som utsattes i Djupasjön nedströms Borås stad och Gässlösa reningsverk, uppvisade relativt höga halter av VTG ($1,81 \pm 0,55$ µg/ml blodplasma) jämfört med referensfiskarna ($0,29 \pm 0,06$ µg VTG/ml plasma). Författarna av rapporten misstänker att källan till problemet är Borås kommunala reningsverk, och inte utsläpp av östrogenliknande ämnen, såsom fenoler. Förllin et al vill inte fastställa om de förhöjda VTG-halterna är en följd av pågående utsläpp eller om de beror av historiska utsläpp som finns lagrade i Viskans och Djupasjöns sediment.

Det kan vara svårt att sätta en fast östrogenkoncentration som ger en viss effekt, eftersom förändringar i hormonella system oftast beror av fler faktorer än koncentration. Bl a påverkar hur lång tid organismen exponerats samt vilket stadium i livet den befinner sig. Ökad syntes av vitellogenin hos foreller har dock rapporterats vid exponering av halter så låga som 0,1 ng östradiolekvivalenter/l [16, 24] och förändringar i könsfördelning vid 0,6 ng/l. 1 ng/l är dock den koncentration där man ser förändringar hos flest fiskarter [12, 24]. Den verksamma dosen som skadar könskörtlarna och hämmar utvecklingen av hanar har ej fastställts för populationer av vild fisk, men antas vara lägre än för induktion av vitellogenin [10]. De olika östrogena substanserna har också olika potential för att utöva östrogen effekt [19]. Både *in vitro* och *in vivo*-försök har visat att den östrogena potensen för E1 är endast en femtedel till hälften så stor som den för E2. Men överlag transporteras flera gånger högre koncentrationer E1 än E2 ut med det utgående avloppsvattnet, vilket gör E1 till den mest betydande, naturliga hormonstörande substansen för vattendjur [4]. E3:s aktivitet ligger $1,3 \cdot 10^{-3}$ gånger längre än den för E2 och många andra, mindre förekommande metaboliter är 10^{-5} - 10^{-7} gånger mindre aktiva än E2 [7]. EE2 anses ha den högsta potensen för att inducera östrogena effekter hos fisk, man tror att substansen kan vara tio gånger så effektivt som E2 på att förorsaka vitellogenin [9]. Anledningen tros vara att naturligt östradiol metaboliseras snabbare än dess syntetiska analog [10]. Mycket talar också för att även halter under detektionsgränserna kan påverka exponerade individer, vilket gör att man inte kan avskriva halter under dessa gränser som helt riskfria.

3. Metod

3.1. Provtagning

3.1.1. Avloppsvatten

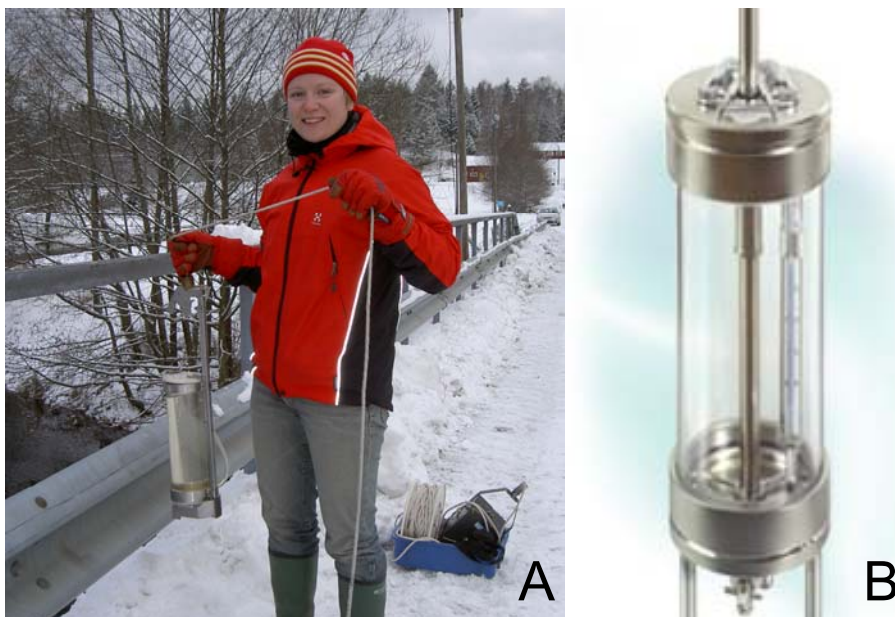
Inkommande och utgående avloppsvatten från Skene avloppsreningsverk (ARV) samlades upp i ett flödesproportionellt 7-dygnsprov, innan det frystes ned. Vatten från Gässlösa ARV samlades i flödesproportionella dygnsprover, som sedan frystes. Dygnsproverna tinades strax innan analys och blandades då proportionellt till det totala flödet under sju dygn.

Provtagningen skedde under en vecka i mitten av mars, 2005. Utförligare beskrivning av provtagningen kan läsas i rapporten om antibiotikarester i vatten och sediment [32].

3.1.2. Recipientvatten

Provtagning av recipientprover skedde mellan 14-17 mars 2005. Vattenproverna togs genom att sänka ned en så kallad Ruttnerhämtare (se Figur 2) från broar ned i mittenfåran av Viskan. Proven förvarades i 1l plastflaskor och förvarades frysta fram till analys. Plats för recipientprovtagningen var främst beroende av läget för broarna över Viskan, eftersom åns branta stränder gör det svårt att samla vattenprover från många platser.

Provtagningspunkterna lokaliserades så att man får en bild av hormonhalterna före utsläpp från reningsverken i Skene och Borås, samt eventuella, detekterbara halter mellan verken och vid Viskans utlopp i Kattegatt, se Figur 3.



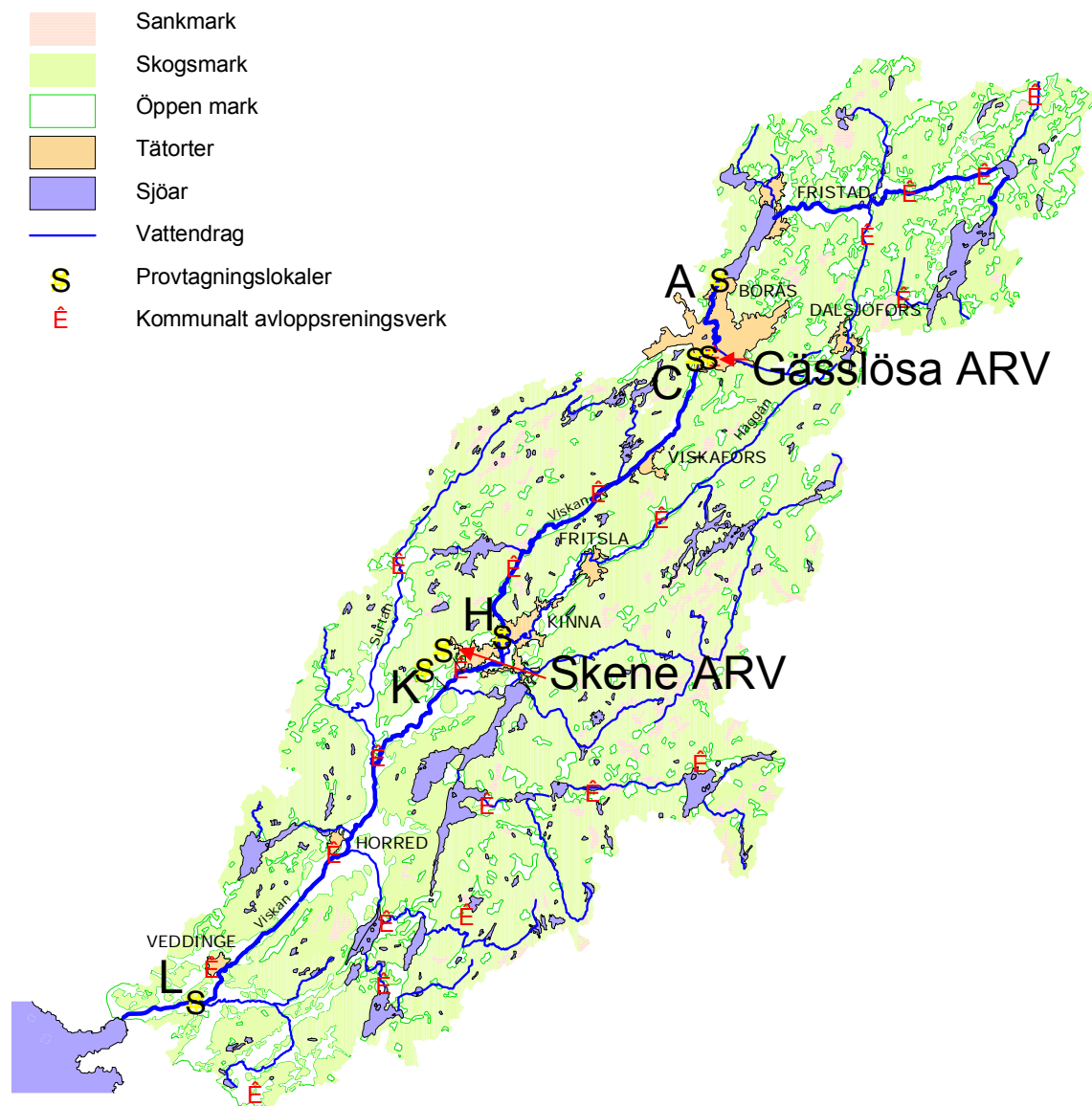
Figur 2. Provtagning av vatten från Viskan (A). Ruttnerhämtare med inbyggd termometer (B).

3.2. Analys av substanserna i vattenprover

Den kemiska analysen av vattenproverna utfördes av Analycen. För närmare information, kontakta Patrik Karlsson, ansvarig för analysen.

De analyserade ämnena har varierande detektionsgräns, beroende på substans och matris (ursprung). Mycket smutsiga vatten, t ex reningsverkens inlopp, medför ofta högre detektionsgränser än mindre smutsiga vatten. Detta förklarar varför detektionsgränsen för

östron, östradiol, östron och etinylöstradiol är 1 ng/l i alla prover, 2 ng/l för norethindron i recipientvatten, 10 ng/l i utgående avloppsvatten och 30 ng/l för samma ämne i inkommande avloppsvatten.



Figur 3. Karta över Viskans avrinningsområde med kommunala reningsverk och provtagningslokaler utsatta. A – Öresjö, före vattenverkets inlopp, Gässlösa avloppsreningsverk, C – Jössabron, H – Kinnaström, Skene avloppsreningsverk, K – Viskadal och L – Åsbro.

4. Resultat och diskussion

I Tabell 5 presenteras de analyserade halterna hormonsubstanser vid respektive provtagningsplats. Om man jämför dessa koncentrationer med de man tidigare uppmätt i Sverige och internationellt, kan man se att halterna i Viskan, samt Skene och Gässlösa reningsverk inte avviker från andra resultat (jämför med Tabell 3 och 4). Halterna ligger inom de som uppmätts i flera Svenska reningsverk [9]. Halten östriol kan anses hög i det inkommande avloppsvattnet, men det finns få resultat att jämföra med, vilket gör det svårt att

säga att halten är ovanligt hög. De finns också få undersökningar av halten norethindron i reningsverk och i naturen, vilket gör det svårt att säga om denna substans ofta påträffas i analyser av dessa miljöer. Överlag är intresset för att analysera progesteroner lågt. Endast östron kunde detekteras i recipienten, vilket inte är något ovanligt scenario. Denna substans påträffas ofta i vattendrag där avloppsvatten släpps ut, i koncentrationer runt de som uppmätts i Viskan. Att östron inte kan detekteras i Öresjön var förväntat, eftersom detta fungerar som referenspunkt där påverkan från antropogena ämnen ska vara som lägst. Det är också här man hämtar det vatten som blir dricksvatten i Borås.

Tabell 5. Uppmätta halter av östron (E1), östradiol (E2), östriol (E3), etinylöstradiol (EE2) samt norethindron (Nor) i Gässlösa och Skene reningsverk (ARV) samt i Viskan [ng/l].

Reningseffekt av vardera substans i reningsverken, uttryckt i procent.

Provtagningsplats	E1	E2	E3	EE2	Nor
Gässlösa ARV, inkommande vatten	32	5,3	129	<1	<30
Gässlösa ARV, utgående vatten	31	6,7	20	<1	<10
<i>Reningseffekt</i>	3%	-26%	85%	-	-
Skene ARV, inkommande vatten	29	12	119	<1	<30
Skene ARV, utgående vatten	1,5	<1	<1	<1	<10
<i>Reningseffekt</i>	95%	>90%	>99%	-	-
Öresjö före inlopp (innan Gässlösa ARV)	<1	<1	<1	<1	<2
Jössabron (efter Gässlösa ARV)	2,8	<1	<1	<1	<2
Kinnaström (innan Skene ARV)	3,4	<1	<1	<1	<2
Viskadal (efter Skene ARV)	2,5	<1	<1	<1	<2
Åsbro (Viskans utlopp)	1,3	<1	<1	<1	<2

Varför koncentrationen av östron fluktuerar mellan provtagningspunkterna kan bero på både påverkan från mindre reningsverk med sitt utlopp i Viskan, samt eventuell påverkan från jordbruket. Man hade kunnat förvänta sig högst koncentrationer strax efter Skenes och Gässlösas utlopp, och något lägre halter däremellan, p g a sedimentering av substanserna, nedbrytning samt utspädning av åvattnet. Förhöjda halter vid Jössabron beror troligtvis av förorening från reningsverket, eftersom denna provtagningspunkt ligger endast ca 500 m nedströms Gässlösa ARV. Samma argumentation gäller för den förhöjda halten vid Viskadal provtagningsplats, som ligger nedströms Skene ARV. Däremot bör, som nämnts tidigare, den detekterade koncentrationen östron vid Kinnaström bero av påverkan från mindre reningsverk belägna mellan Gässlösa och Skene ARV.

Halterna för de andra hormonella substanserna, d v s E2, E3, EE2 och norethindron, ligger under 1 ng/l respektive 2 ng/l, vilket tyder på att dessa ämnen antingen bryts ned snabbt, eller lämnar reningsverken i så låga koncentrationer att de inte är detekterbara. Det är positivt att se att koncentrationen av främst EE2, som är ett mycket potent hormon, inte är detekterbar. Man kan dock inte, om man får tro vad Svenson påstår [9], helt avvisa halter under detektionsnivån som icke skadliga, eftersom det kan tänkas att lägre koncentrationer kan ge negativa effekter hos levande organismer. Detektionsnivån för dessa ämnen i recipientvatten är ju också den koncentration där man ser negativa effekter hos flest fiskar [12, 24]. Man kan därför inte, endast utifrån de kemiska analyserna, dra slutsatsen att fiskar i Viskan är helt opåverkade av hormonella substanser. För att se om negativa effekter hos fiskarna verkligen föreligger, måste man komplettera de kemiska analyserna med biologiska test. Genom att utföra *in vivo*-

test, där man utsätter levande organismer för den rådande miljön, kan man upptäcka om ett vattendrag innehåller nog höga halter östrogena substanser för att ge effekter hos organismer. Ett sådant försök har IVL gjort, då man satte ut fiskar i burar nedströms bl a Skene och Gässlösa reningsverk [9, 10]. Dessa undersökningar uppvisade inga effekter hos fiskarna, vilket gör att man drar slutsatsen att halterna av östrogena substanser fortfarande är så låga i Viskan att de inte kan ses som skadliga för de fiskar som lever däri. Den undersökning som utförts av Zoofysiologi, Göteborgs Universitet, visar dock att Viskan innehåller så pass höga halter hormonella substanser att VTG-halten är förhöjd. Författarna bedömer situationen så pass allvarlig att det kan innebära att fiskarna som uppehåller sig i området har försämrade reproduktionsförmåga. Vidare diskuterar man om de förhöjda VTG-koncentrationerna beror av pågående utsläpp, eller härstammar från äldre utsläpp som samlats i sedimenten. Man har ju detekterat östrogena substanser i flodsediment (se Tabell 4) och även sett att fiskar som utsatts för kontaminerade sediment har uppvisat hormonella störningar [1, 3]. I universitetets undersökning såg man högst VTG-halt i de fiskar som satts ut i Djupasjön, vilken är känd för att hysa mängder av föroreningar som härstammar från bl a textilindustrins forna utsläpp. Att östrogena substanser från reningsverken sedimenterat här, är ingen omöjlighet, eftersom ämnens egenskaper tillåter inbindning till sediment. De höga vitellogeninhaltarna kan därför härröra från förorenade sediment vid provtagningsplatsen. Rent teoretiskt borde fiskarna i IVL:s undersökning uppvisa högre halter VTG i blodet, än de fiskar som använts i universitetets försök, om hormonkoncentrationerna i Viskans vatten var ganska lika mellan de olika tidpunkterna för provtagning. Detta eftersom fiskarna i IVL:s test satts ut i burar närmare reningsverket, där hormonkoncentrationerna borde vara högre, och dessutom under längre tid. Det är dock svårt att dra generella slutsatser av dessa försök, eftersom de aldrig kan imitera verkligheten, dessutom kan effekterna variera mellan fiskart, provtagningsplats och tidpunkt. Eftersom resultaten från IVL och Zoologiska institutionen uppvisar så tydliga skillnader, är det svårt att säkerställa om avloppsvattnet från de kommunala reningsverken negativt påverkar de fiskar som lever i Viskan, eller inte. Förlin et al rekommenderar ytterligare undersökningar av reningsverkens avloppsvatten för att avgöra om fiskarna i området har nedsatt reproduktionsförmåga.

Analysen av vattenproverna visar att hormonhalten i Gässlösa generellt är något högre än i Skene reningsverk, både vad gäller inkommande och utgående avloppsvatten. Koncentrationen av östradiol ökar till och med i Gässlösa, vilket tyder på att det sker en omvandling av konjugerade metaboliter till den aktiva formen. Dessutom är reduktionen av östron mycket låg, vilket också kan bero på dekonjugering och omvandling av utsöndrade metaboliter. En tidigare undersökning av dessa två reningsverk, visade även den att vattnet i Gässlösa hade högre koncentrationer östradiolekvivalenter än Skene [17]. Det finns inga enkla svar vad denna skillnad beror på – eventuellt kan en större inblandning av avloppsvatten från andra källor än hushåll, t ex textilindustrin, i Skene ge en lägre koncentration hormoner. Det är dock positivt att varken etinylöstradiol eller norethindron går att detektera i något av verken.

Reningseffekten i Gässlösa är också försämrade jämfört med reduktionen i Skene, som uppvisar mycket hög avlägsnandegrad av de analyserade substanserna. Det är svårt att dra en enkel slutsats om vad detta beror på, eftersom de båda verken utnyttjar liknande reningsmetoder [33, 34]. De två processer som uppvisat ge högst avlägsnande av hormoner – aktivt slam och kväverening – finns representerade i båda verken. Man kan dock i dessa undersökningar inte visa att det finns en, eller flera kombinerade, reningsmetoder som utan undantag ger ett högt avlägsnande av östrogena ämnen, vilket gör det svårt att ge ett generellt råd om vilka processer som ska användas eller modifieras i ett reningsverk för att man ska

uppnå hög reduktion av östrogena ämnen. Ett och samma reningsverk har ju dessutom uppvisat varierande reduktion av de aktuella ämnena vid olika provtagningstidpunkter [10]. Teoretiskt sett, skulle därför reningseffekten i Skene och Gässlösa kunna vara lika, men Gässlösa uppvisade vid tidpunkten för denna provtagning försämrade reduktion av hormoner. Det som talar för att en skillnad verkligen föreligger mellan verken, är, som nämnts ovan, att en annan undersökning också påvisat att Gässlösa uppvisar högre hormonkoncentrationer än Skene [9]. Två undersökningar som visar på samma resultat är svårt att avfärda, varför man bör dra slutstansen att det finns en signifikant skillnad i reningseffekt mellan den två verken.

Man har också sett att längre uppehållstid för vattnet i de biologiska reningsstegen samt upprepade fällningssteg kan eventuellt också ge förhöjd reduktion av de aktuella substanserna. Antalet fällningssteg bör dock inte vara en avgörande orsak i detta fall, eftersom Gässlösa reningsverk begagnar sig av fler steg än Skene ARV. Beträffande uppehållstiden i det biologiska reningssteget, ligger den i Skene närmare 13,5 timmar och i Gässlösa strax över 9 timmar [35]. Denna tidsskillnad kan eventuellt bidra till den högre avlägsnandegraden i Skene reningsverk, eftersom avloppsvattnet kommer i kontakt med nedbrytande bakterier under längre tid där, vilket kan leda till högre nedbrytning av de hormonella ämnena. Detta är den enda undersökta parametern som eventuellt kan förklara varför reningsverket i Skene lyckas bättre med att bryta ned och avlägsna de östrogena substanserna som transporters med avloppsvattnet.

Den försämrade reningseffekten i Gässlösa reningsverk har dock inte lett till detekterbart högre koncentrationer hormon i recipienten. Kanske går det att visa högre halter hormon i Viskan strax efter utloppet från Gässlösa jämfört med utloppet från Skene, men troligtvis är halterna för låga och utspädning för stor för att se skillnader mellan dessa tänkta provtagningstidpunkter.

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