Monitoring anti-infectives and antibiotic resistance genes - 
with focus on analytical method development, effects of antibiotics and national perspectives

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Department of Chemistry
Doctoral Thesis 2012
Dedicated to my beloved parents
List of papers

This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals.


II Ghazanfar Ali Khan, Björn Berglund, Stefan E.B. Weisner, Per Magnus Ehde, Per-Eric Lindgren, Jerker Fick. 2012. At environmentally-relevant concentrations, antibiotics do not affect bacterial community patterns in constructed wetlands. *Submitted to Ecological Engineering*


V Andrew C. Singer, Josef Järhult, Roman Gracic, Ganna Fedorova, Ghazanfar Ali Khan, Jerker Fick, Richard H. Lindberg, Michael J. Bowes, Björn Olsen, Hanna Söderström. 2012. Compliance to Oseltamivir among two populations in Oxfordshire, United Kingdom affected by Influenza A(H1N1)pdm09, November 2009 – a wastewater epidemiology study. *Submitted to PLOS One*

Paper I is reproduced with kind permission from Elsevier.

**Contribution by the author of this thesis to the papers**

The author was responsible for the followings: planning of study (*Papers I, II, III and IV*); sampling (*Papers I, and IV*); sample handling (*Papers I, II, IV and V*); experimental work and analysis (*Papers I, II, IV and V*); evaluation and interpretation of the data (*Papers I, II, IV and V*); and writing the papers (*Papers I, II and IV*).
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARG</td>
<td>Antibiotic resistance gene</td>
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<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple drug resistance</td>
</tr>
<tr>
<td>WWTP</td>
<td>Waste water treatment plant</td>
</tr>
<tr>
<td>DPF</td>
<td>Drug production facility</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometer</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>CW</td>
<td>Constructed wetland</td>
</tr>
<tr>
<td>AR</td>
<td>Antibiotic resistance</td>
</tr>
<tr>
<td>BDM</td>
<td>Bulk drug manufacturer</td>
</tr>
<tr>
<td>van A</td>
<td>Vancomycin resistance gene A</td>
</tr>
<tr>
<td>mec A</td>
<td>Methicillin resistance gene</td>
</tr>
<tr>
<td>HESI</td>
<td>Heated electron spray ionization</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denatured gradient gel electrophoresis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>ESI</td>
<td>Electron spray ionization</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Cycle of threshold</td>
</tr>
<tr>
<td>van B</td>
<td>Vancomycin resistance gene B</td>
</tr>
<tr>
<td>qnr S</td>
<td>Quinolone resistance gene</td>
</tr>
<tr>
<td>tet A</td>
<td>Tetracycline resistance gene A</td>
</tr>
<tr>
<td>tet B</td>
<td>Tetracycline resistance gene B</td>
</tr>
<tr>
<td>erm B</td>
<td>Macrolide, lincosamides and streptogramin resistance gene</td>
</tr>
<tr>
<td>dfr 1</td>
<td>Trimethoprim resistance gene</td>
</tr>
<tr>
<td>intI 1</td>
<td>Integrase on class 1 integrons</td>
</tr>
<tr>
<td>SULM</td>
<td>Sulphamethoxazole</td>
</tr>
<tr>
<td>TRI</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>OXY</td>
<td>Oxytetracyclin</td>
</tr>
<tr>
<td>CIP</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>OC</td>
<td>Oseltamivir carboxylate</td>
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</table>
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1. Introduction

Today it is hard to imagine the pre-antibiotic era, when most bacterial diseases were life threatening. Nevertheless, during the past century, one of the biggest progresses in human and animal health is the discovery and usage of penicillin and other classes of antibiotics during 1940s to 1960s. This consequently resulted in rapid decline in the death rate caused by infectious diseases and brought about overall improvement of health care.

Antibiotics are biologically active and are widely used in humans and animal medicine to prevent or treat microbial diseases. In addition to their clinical uses, some of them, albeit in sub-therapeutic amounts, are still in use as growth promoters, feed additives in livestock management, poultry and fish farming in various parts of the world. The growing use of antibiotics for therapeutic and non-therapeutic purposes has also led to the development and dissemination of microbial resistance both in clinical and environmental settings. Globally, there has been growing concern regarding increased presence of antibiotic residues in the aquatic and terrestrial environments. The particular concern is the potential development of microbial resistance in the presence of very low antibiotic concentrations and further promotion and spread of antibiotic resistance genes (ARGs) to human pathogens via horizontal gene transfer (HGT) facilitated by mobile genetic elements such as plasmids, transposons and integrons. The pathogens carrying ARGs could end up in the food web and subsequently carried to humans and animals thus causing diseases and treatment failure.

The ongoing indiscriminate misuse and overuse of antibiotics in humans, animals and agriculture is the cause of increasing trend in microbial resistance. Resistance to antibiotics first appeared in hospitals with the emergence of sulfonamide-resistant Streptococcus pyogenes in 1930s followed by penicillin-resistant Staphlococcus aureus in the 1940s [1]. However the biggest threat is posed by multiple drug resistance (MDR) and some pathogens such as Mycobacterium tuberculosis, methicillin-resistant Staphlococcus aureus (MRSA), Vancomycin-resistant Staphlococcus aureus (VRSA), Vancomycin-resistant enterococci (VRE), Klebsiella pneumonia, Pseudomonas aeruginosa and Acinetobacter baumannii are the global notable examples in the hospitals and community [1].

Antibiotics as humans and animals’ excretory products (active, metabolites and conjugates) enter into water bodies either directly or after passage
through waste water treatment plants (WWTPs) [2]. In addition to this they are also released into environments as inappropriately disposed of unused drugs and in effluent of drug production facilities (DPFs) [3]. They have been detected in surface water, ground water, drinking water, biota, and sediments at levels ranging from less than 1 ng/L up to a few µg/L [2].

The detection of trace antibiotics has always remained a challenge; nevertheless it has been achieved via the improvement and advancement in detection technologies. However, the most significant contribution has been with mass spectrometry (MS), which has become a technique of choice for monitoring levels of antibiotics [4]. Liquid chromatography coupled to mass spectrometry (LC-MS) and LC-MS/MS has obvious advantages over gas chromatography coupled to mass spectrometry (GC-MS) as it could be used for measurement of wide range of polar compounds and does not require lengthy and irreversible derivatization which is sometimes necessary in GC-MS analysis. Normally samples undergo enrichment step by solid phase extraction (SPE). A significant addition is the usage of automated on-line SPE system coupled to LC-MS/MS which makes the enrichment of compounds in a more rapid, precise and efficient manner than the labor-intensive and time-consuming conventional techniques. Furthermore, it has an advantage of high sample throughput, minimal solvent utilization and low sample preparation times and has been used in the analysis of biological and environmental samples of various classes.

Waste water treatment plants (WWTP) do not always remove these compounds efficiently and therefore, to further enhance the efficiency of removal, a number of sophisticated and useful treatment technologies have been developed such as ozonization, ultrafiltration, advanced oxidation, treatment with activated carbon and UV/H₂O₂, however, these are relatively expensive [5]. Studies show that constructed wetlands (CWs) have the potential to serve as a promising low-cost alternative to conventional tertiary treatment methods for removing or reducing levels of nitrogen, phosphorous, pharmaceuticals including antibiotics and the biological oxygen demand (BOD) through natural processes by plants, microorganisms, solid matrix components and sunlight [6-8]. Nevertheless, introduction of high concentration of antibiotics could subsequently affect the functionality and water purifying properties of CWs.

Furthermore, CWs have a potential risk of becoming a breeding ground for resistant bacteria either due to introduction of resistant bacteria in the waste water effluent or via antibiotic selective pressure; in either case, the ARGs are capable of disseminating via HGT [9, 10].
1.1 Objectives and aim

During recent decades, the sources, occurrence, fate and effects of antibiotics, in particular resistance development have been in scientific and public focus. However, greater knowledge is required regarding their effects in CWs. The main objective of the study underlying this thesis is to develop a rapid on-line method for analysis of antibiotics, to understand the relationship between environmentally-relevant antibiotic concentration effects on bacterial community composition and resistance development in CWs. Furthermore, to investigate the occurrence of anti-infectives and ARGs in various water bodies. More specific aims reported in Papers I – V are:

1. To develop and apply sensitive, efficient and reliable methods for the simultaneous determination of anti-infectives (I, II, IV and V).
2. Investigate the effect of environmentally-relevant concentrations of antibiotics on the bacterial composition and occurrence of antibiotic resistance genes (II, III and IV).
3. Investigate the sources, occurrence and abundance of antibiotics and resistance genes in various water bodies (IV).
4. Back calculate the compliance and consumption of oseltamivir in populations during influenza pandemic (V).
2. Target anti-infectives and antibiotic resistance

Antibiotics are biologically active compounds produced by microorganisms, semi-synthesized or synthesized, possessing therapeutic activity to kill or inhibit the bacteria, fungi or protozoa whereas the term ‘antimicrobial’ broadly refers to any antibiotic, food antimicrobial agent, sanitizer, disinfectant which acts against microorganisms including viruses. They are not only widely used to treat infections in humans and animals but are also used as therapeutic, prophylactic and growth promotion agents in veterinary, agriculture and aquaculture practices [11].

Before the antibiotic era, derivatives of heavy metals or arsenic were in use for treatment of infections. The first groups of antimicrobials were sulfonamides which were introduced in the mid-1930s, however, soon their use was restricted due to appearance of resistant strains of streptococci and Gram-positive pathogens. Penicillin, the antibiotic of natural origin produced by the fungi of the genus Penicillium was first discovered by Alexander Fleming in 1928. However, it was later with the efforts of Howard Florey (1898-1968) and Ernst Chain (1906-1979) that therapeutic use in humans initiated during 1941-1942. The mass production of penicillin in 1943 and wider use for treatment of infections marks the advent of antibiotic era. Furthermore, the remarkable ability of antibiotics in controlling infections and consequently achievement of rapid decline in death rates due to infections during and after Second World War led to the isolation of other major classes of antibiotics during the 1940s to 1960s [12]. The brief overview of history of antibiotics and development of resistance are given by Zaffiri et al. 2012 [13] and Taubes et al. 2008 [14] and is presented in figure 2.1(a) and (b).

Antibiotics can be classified into groups based on similar chemical structure and chemical behavior or on the basis of same mode of action e.g. β-lactam and quinolones.
Figure 2.1(a). Timeline of antibiotics history – usage

Figure 2.1 (b). Timeline of antibiotics history – resistance.

Methicillin resistant staphlococcus aureus, Vancomycin resistant enterococcus, Staphlococcus aureus with intermediate resistance to vancomycin, Community acquired–MRSA, Linezolid–resistant staphlococcus aureus, Vancomycin resistant staphlococcus aureus
2.1 Groups of antibiotics and chemical structures

Quinolones and fluoroquinolones are synthetic compounds that inhibit the bacterial DNA synthesis and replication. Nalidixic acid remained for a long time a drug of choice for urinary tract infections. Other members of the class such as ciprofloxacin and norfloxacin are broad-spectrum bactericidal antibiotics.

Tetracyclines, macrolides and lincosamides are protein synthesis inhibitors. Tetracyclines act by binding to the specific sites in the 30S subunit of bacterial ribosome while others bind to the 50S subunit. Tetracyclines are broad-spectrum antibiotics and are active against both gram positive and negative bacteria, however, broadly speaking they are bacteriostatic. Moreover they are effective against Lyme disease and some sexually transmitted bacterial diseases (STDs) such as chlamydia and gonorrhea. They are also widely used in veterinary medicine and in animal feeds to improve growth efficiency. Macrolides are bactericidal against some gram positive bacteria and its member, azithromycin is effective against STDs as mentioned above. Lincosamides are effective against obligately anaerobic bacteria such as Bacteroides species and protozoa such as Giardia.

Sulfonamides and trimethoprim are inhibitors of tetrahydrofolate biosynthesis. Sulfonamides is structurally similar to p-aminobenzoic acid and competitively inhibit the the first enzyme whereas trimethoprim is structurally similar to dihydrofolic acid and thus competitively inhibits the dihydrofolate reductase. When used alone they are bacteriostatic but in combination they are bactericidal. They are also used in animal husbandry.

β-lactams (penicillins, cephalosporins, carbapenems, monobactams) and glycopeptides (vancomycin, daptomycin, teichoplanin) are peptidoglycan synthesis inhibitors and thus inhibit the cell wall synthesis. Some β-lactam antibiotics are more effective against both gram positive and gram negative while others are more effective against gram positive than gram negative.

Vancomycin and daptomycin are clinically very important as they are used as last resort drug for the treatment of some resistant gram positive pathogens such as S.aureus and Enterococcus species.

Oseltamivir is an antiviral belonging to the class of neuroaminidase inhibitor and is used for the treatment of seasonal flu. Its prodrug, oseltamivir phosphate is converted to active metabolite, oseltamivir carboxylate in the liver and excreted via urine.
Antibiotics have complex molecular structures with different functional groups, thus, having different physicochemical (solubility, hydrophobicity, hydrophilicity, log $K_{ow}$ or log $K_D$) and biological properties under different pH conditions due to existence in the neutral, cationic, anionic or zwitterionic forms, e.g. ciprofloxacin has both acid and basic functional groups with acid constants at 6.16 and 8.63 respectively and isoelectric point at pH 7.04 [11]. Similarly, tetracycline has several ionizable functional groups with varying charges depending upon the pH of the solution such as tricarbonyl methane ($pK_a$ 3.3); dimethyl ammonium cation ($pK_a$ 9.0); and the phenolic diketone ($pK_a$ 7.7) [15].

The compounds for the paper I, II and III are selected on the basis of their stability, environmental impact and consumption in Sweden and for paper IV and V are based on their usage in Pakistan and U.K respectively. The chemical structure and groups are given in Table 2.1.
Table 2.1. Chemical structures and groups of antibiotics.

Fluoroquinolones

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>R₁</th>
<th>R₂</th>
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<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>cyclopropane</td>
<td>H</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>C₂H₅</td>
<td>H</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>cyclopropane</td>
<td>C₂H₅</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td>C₂H₅</td>
<td>CH₃</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enoxacin</td>
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</tr>
</tbody>
</table>

Tetracyclines

<table>
<thead>
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<th>Antibiotic</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>OH</td>
<td>CH₃</td>
<td>H</td>
</tr>
</tbody>
</table>

Sulphonamides

<table>
<thead>
<tr>
<th>Antibiotic</th>
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<th></th>
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<tbody>
<tr>
<td>Sulfamethoxazole</td>
<td>NH₂</td>
<td></td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>NH₂</td>
<td></td>
</tr>
</tbody>
</table>
Dihydrofolate Reductase Inhibitor

Trimethoprim

Macrolides

Erythromycin: $R_1 = \equiv \text{O}$, $R_2 = \text{OH}$
Clarithromycin: $R_1 = \equiv \text{O}$, $R_2 = \text{OCH}_3$
Roxithromycin: $R_1 = \equiv \text{N} - \text{O} - \text{O} - \text{CH}_3$

Lincosamides

Clindamycin
Lincomycin

Cephalosporin

Antiviral

Cefotaxime
Oseltamivir
2.2 Antibiotic resistance

Antimicrobial resistance (AR) is the relative insusceptibility of a microorganism to a particular treatment of antibiotics under a particular set of conditions and can be quantified using the minimum concentration inhibiting the growth of microorganisms. The microorganisms exhibiting the change in susceptibility are therefore considered as resistant. Some microorganisms are intrinsically resistant to antibiotics due to their physiology or biochemistry while others acquire resistance either via mutation or by resistance genes.

Although AR existed even in pre-antibiotic era, it got recognition with the clinical use of antibiotics, primarily in hospitals where they were first introduced to cure infections. Consequently resistance appeared to sulfa drugs in 1930 and then later to penicillins in 1940. AR kept on increasing with the growing use of antibiotics until 1970, when it was viewed as threat with the emergence of number of MDR microorganisms both in the hospitals and community.

Lately, AR in the environment also got attention from the scientific community with the realization that ARGs can be shared among the other, same or far related bacterial species. Of course this has very serious public health consequences as the bacteria of little importance can easily transfer ARGs to the pathogens causing disease in people.

Over the last 60 years as a consequence of the use of antibiotics a specific number of ARGs out of large number of resistant determinants present in natural ecosystems have spread among human pathogens [16].

2.2.1 Modes of action and mechanisms of resistance

The modes of action for target antibiotics and mechanism of resistance in general are given in Figure 2.2.

Modes of action are namely inhibition of cell wall biosynthesis (e.g. penicillins, cephalosporins), inhibition of DNA-biosynthesis and reproduction (ciprofloxacin), inhibition of folate synthesis (e.g. sulfamethoxazole, trimethoprim) and inhibition of protein biosynthesis at ribosomal level (e.g. macrolides, tetracyclines, lincosamides) [17].
The mechanism of drug resistance in general are limiting intracellular accumulation due to changes of outer membrane permeability, decreased influx (inner membrane transport) or active efflux, enzymatic modification or the degradation of the antibiotic via hydrolases (β-Lactams), kinases (aminoglycosides, macrolides) and acyltransferases (aminoglycosides, fluoroquinolones), target modification by mutation in ribosomal proteins or in 16S rRNA, target bypass by production of alternate cell wall (glycopeptides), sequestration, biofilm formation, and target amplification.

**Figure 2.2** Modes of action and mechanisms of resistance
3 Sources, occurrence, fate and effects of antibiotics and antibiotic resistance genes

The consumption and usage patterns of antibiotics vary from country to country depending upon prescribing and agricultural practices and the local regulations. In some countries they are only available on prescription, while in others they are freely available over the counter. In some they are widely used in animal husbandry or as growth promoters while in others this usage is banned or limited therefore the sources, occurrence, fate and effects of antibiotics vary.

3.1 Sources

3.1.1 Usage for treatment, households and hospitals

Antibiotics are used in large quantity for the treatment of humans and animals and are primarily released into the environment through wastewater, see figure 3.1. Antibiotics are released via urine and faeces, either in unaltered form, or as metabolites / conjugates after action of enzymes in the liver, hydrolysis in the stomach or breakdown by the bacteria inhibiting the surface of the skin [11, 18]. Excretion rates for intact antibiotic compound varies from 10% - 90% however, Kummerer and Henninger, 2003 [19] estimated the metabolic rate to be 30% and intact antibiotic compound excreted to be 70%, if the average volume for all antibiotics used is totaled.

Pharmaceuticals including expired or unused left over antibiotics are sometimes disposed of down household drains either via sink or toilet. A study carried out in U.S, reported that half of the patients flush drugs down in toilets and only 22.9% returned them to pharmacies [20].

Though higher levels of antibiotics are observed in hospital effluents [21, 22] owing to high usage and less dilution as compared to household effluent; in reality they are not the main source of antibiotics into municipal sewage as their contribution accounts for only 5-20% of the total antibiotic use. Moreover, in UK, USA and Germany community usage of antibiotics is far higher (≥ 70%) than in hospitals [23]. Nevertheless, hospitals are the major source of antibiotics for the cephalosporin group of antibiotics on account of their frequent usage to control infections [19].
Figure 3.1. The sources of antibiotics in the environment
3.1.2 Animal husbandry, aquaculture and agriculture

Antibiotics, worldwide are used in small amounts as growth promoters in feed with aim to improve the quality of meat with lower percentage of fat and higher percentage of protein content [24]. They are excreted from animals and are spread with manure to the soil, which then may reach surface water through run-off. However, in some European countries owing to development of resistance in bacteria they are banned as growth promoters.

In aquaculture or fish farming, antibiotics are used as prophylactic agents to enhance the growth of fish and consequently are the direct source of antibiotics into the aquatic environment.

Antibiotics such as streptomycin with oxytetracycline are used in small quantities to control certain bacterial diseases in fruits (apple and pear), vegetables and ornamental plants [11]. The application to fields and subsequent run-off also adds to the sources via agriculture usage.

3.1.3 Drug production facilities

One of the largest point source, which has been until now unnoticed, is discharges from bulk drug manufacturers (BDM). Larsson et al; [3] reported that the effluent from a treatment plant receiving water from approximately 90 bulk drug manufacturing industries contained antibiotics with levels of fluoroquinolone, ciprofloxacin observed as high as 31 mg/L in the effluent which is equivalent to 45 Kg or 45000 daily doses. Later Fick et al. 2009 [25] reported 14 mg/L concentration of ciprofloxacin in the same treatment plant which indicates that the situation was still on-going. Li et al. 2008 [26] observed concentrations of 19 mg/L of oxytetracycline in treated effluent from a Chinese antibiotic factory which is approximately 20 times the human therapeutic plasma levels. The discharges from BDM are common in Asian continent, however, Europe [21] and USA [27] are also contributing.

3.2 Occurrence

As a result of partial removal, antibiotics pass through sewage treatment plants (STPs) and consequently end up in the surface waters, groundwater and sediments, see figure 3.1. They also enter directly into environment via agriculture and animal husbandry related activities. Various classes of antibiotics including penicillins, macrolides, aminoglycosides, tetracyclines, sulphonamides, and quinolones have been detected in more or less same range in the higher microgram-per-liter range in hospital effluent, in the lower microgram-per-liter range in municipal wastewater, and in the higher and lower nanogram-per-liter range in different surface waters, ground water
and seawater [28-32]. In general a decreasing order of antibiotic concentrations are found in; municipal sewage, hospital effluents, influent and effluent of STPs, surface water, groundwater and sea water.

Antibiotics have been detected in sediments in the range of low ng/L to low µg/L. Kim and Carlson (2007) [33] measured tetracyclines, sulphonamides and macrolides in relatively higher concentration in sediments than in the surrounding water matrix in agriculturally impacted river. Moreover in aquaculture antibiotics are added directly into the water which consequently settles into the sediments resulting in higher local concentrations.

In addition to sediments [34], antibiotics have also been found to occur in plants especially following the application of manure containing antibiotics. Few studies reported the concentration of antibiotics in vegetables up to low mg kg\(^{-1}\) dry weight [11].

### 3.3 Fate

Antibiotics are eliminated from the environment by abiotic processes such as sorption to the soil or sediment particles, photolysis, hydrolysis, thermolysis and oxidation in the STPs and biotic process i.e. biodegradation via bacteria and fungi.

#### 3.3.1 Sorption

Elimination of antibiotics by the sorption depends upon the physico-chemical properties of the antibiotics, distribution coefficients (\(k_d\)), the mineral content of the soil, sediment and sludge, thus contributing to their removal, spread and (bio)availability in the environment. The formation of complexes of antibiotics with particles also lowers the antibacterial activity of the compound i.e tetracyclines form complexes with double cations (Mg and Ca) [35]. Complexation is also affected by the presence of humic substances which can either increase or decrease the sorption via altering the surface properties of the particles.

#### 3.3.2 Photolysis, hydrolysis and thermolysis

Depending upon the intensity and frequency of light, certain antibiotics undergo photochemical decomposition in clear surface water. Quinolones, tetracyclines, and sulphonamides are photosensitive, therefore oxytetracycline is reported to be stable in sediments as compared to sea water. Furthermore fluoroquinolones are degraded by UV light [36].

\(\beta\)-lactam antibiotics are prone to hydrolysis whereas sulphonamides and quinolones are resistant. Some tetracyclines are unstable in water to certain
extent and in particular oxytetracycline hydrolysis rate increases as pH deviates from 7 with increasing temperature [11].

3.3.4 Oxidation and biodegradation in STPs

Although advanced treatment technologies used as tertiary treatment of effluent in WWTPs are effective but they are expensive. Ozonization has been used to degrade oxytetracycline and sulfamethoxazole. Biodegradation primarily by bacteria and fungi is the biotic process of removal of antibiotics. Sewage system has a higher biodegradation rate than the surface water due to higher density, diversity and pre-adaption of bacteria. In general various studies have reported that antibiotics are not readily biodegraded [11].

3.4 Effects

Antibiotics have the potential to affect microorganisms in both the aquatic and terrestrial environment. They are present as mixtures in the environment and even single antibiotic with its transformed products and metabolites could be considered as multi-component mixture. Some antibiotics have similar mode of action and thus could have additive affect, therefore antibiotics mixture effects on bacterial community in the environment are of great significance. Number of studies has reported the ecotoxicological effects of antibiotics in Daphnia, algae and bacteria at much higher concentrations (g/L) than generally present in the environment; however, the lower concentration exposure for a longer duration of time can produce similar effects as with of higher concentration [37].

Antibiotics could affect degradation in STPs by inhibiting the waste water bacteria. Furthermore they could also affect the nitrifying bacteria, algae and micro-algae in surface water. Nevertheless, one of the most important effect of antibiotics is the development of resistance in aquatic bacteria.

3.5 Antibiotic Resistance Genes (ARGs)

Antibiotics stimulate the transfer of ARGs and increase the speed of bacterial strains selection when exposed to microorganisms for a long period of time at subtherapeutic concentrations. However knowledge regarding resistance at subinhibitory concentrations is still limited and resistance cannot be correlated with the concentration of antibiotics in the environment. Moreover cross resistance occurs where microorganisms not only acquire resistance by coming in contact to a certain antibiotic but also become resistant to other antibiotics [38].
Still another viewpoint is that resistance genes are already present and exchanged between bacteria in the natural environment or acquired by human pathogens via HGT [39-42]. ARGs are ubiquitous in all ecosystems, either contaminated or pristine, or even in commensal microbiota of wild animals [16, 43, 44].

3.6 Sources of ARGs

There are many sources of ARGs into the environment, see figure 3.2.

3.6.1 Hospital effluent

Antibiotics concentration released into the environment from hospitals is relatively higher due to less dilution of effluent and may reach the same order of magnitude as the minimum inhibitory concentration for sensitive pathogenic bacteria [19], thus, increasing chances of selection, in particular multi-resistant bacteria. VanA [45], and mecA [46] have been found in hospital effluent. In addition to this, gentamicin resistance genes were also found in hospital effluent in Acinetobacter, Pseudomonas and Enterobacteriaceae [46].

3.6.2 Waste water and STPs

ARGs linked to human and animal bacteria along with antibiotics are released into wastewaters from farm, hospitals and cities [9, 42, 47, 48]. Various resistant and multi-resistant bacteria such as E.coli, P.aeruginosa, Acinetobacter, pseudomonads, enterobacteriaceae and phylogenetically distant bacteria, such as members of α- and β- Proteobacteria, have been found in municipal sewage in aeration tanks and the anaerobic digestion process in STPs [49]. Agriculture organic wastes from livestock and sewage sludge from urban effluents contribute to the release of ARGs in natural ecosystems [50, 51]. Resistant and multi-resistant pathogenic bacteria such as Acinetobacter species has been found in waste water and STPs.
**Figure 3.2.** The sources of antibiotic resistance genes in the environment.

### 3.6.3 Surface water, ground water and drinking water

ARGs are present in surface water, marine bacteria, estuaries and coastal waters [49, 52]. Studies showed that correlation exists between the resistant bacteria in rivers and urban water input. Resistant bacteria (*E. cloi*) has been found in rural ground water [53] and in drinking water [46, 54].

### 3.6.4 Sediments and soil

Antibiotics, in particular oxytetracycline, have been applied directly to fish farms which ultimately end up in sediments in high localized concentration thus, providing suitable environment for the selection of microorganisms. Tetracycline-resistant Gram-negative bacteria have been isolated from polluted and unpolluted marine sediments [55]. Presence of resistant bacteria in sediments is considered as an indicator of past antibacterial use. Manure containing veterinary antibiotics such as tetracyclines, some sulphonamides and quinolones are applied as fertilizer to the agriculture land. These antibiotics are strongly sorbed to the soil and ultimately accumulate and select resistant bacteria [49].
3.7 ARGs vs chemical pollutants

It is important to differentiate when resistance can be considered as a result of pollution and when it is a normal state of given ecosystem. Chemical pollutants produces a gradient of contamination in time and space depending upon where pollution occurs (soil, water), the intrinsic diffusion properties (solubility) and degradation. It is expected that if the release of the chemical ceases, pollution will decrease and eventually disappear (although some chemical are very persistent). Antibiotics on other hand can increase resistance which can persist and eventually spread even in the absence of pollution with antibiotics.

ARGs are auto replicative pollutants and their concentration increases under selective pressure thus their maintenance does not depend on their constant release and can diseminate via mobile units to other bacteria. Consequently identical ARGs have been found in disconnected aquatic systems [60].

In addition to this, ARGs are ubiquitous [56] and has been found in the regions where human contact is unlikely such as deep terrestrial subsurface [57], the deep Greenland ice core [58] or the waters of the Antarctic Ocean [59]. However higher amount of ARGs are found in sediments located nearby cities and sewage tributaries than in pristine environments.

Furthermore ARGs can travel long distances via birds, wild animals and even traffic ships can contribute in the dissemination of bacteria between different oceans and continents [61-63]. Additionally our global world is interchanging people and goods on massive scale and resistance emerging in areas of high use of antibiotics will sooner or later spread to comparatively clean environments.
4. Analytical methods

The analytical methods used were on-line solid phase extraction (SPE), offline SPE techniques in conjunction with heated electron spray ionization (HESI) and liquid chromatography mass spectrometry (LC-MS). For molecular biological analyses, denatured gradient gel electrophoresis (DGGE) was used to determine the bacterial community diversity and real time polymerase chain reaction (RT-PCR) was used to determine the antibiotic resistance genes.

4.1 Chemical analysis

All the water samples were prepared by filtration through 0.45 µm filters; MF<sup>TM</sup> – membrane filter (MF, Millipore, Syndberg, Sweden) for the offline SPE (Paper II) and syringe driven filter units 0.45 µm (Millipore) attached to the syringes in the case of on-line SPE/LC-MS-MS (Paper I, IV and V).

![Diagram of solid phase extraction process](image)

**Figure 4.1. Procedure of solid phase extraction.**

4.1.1 SPE

Solid phase extraction is used for sample cleanup and to concentrate the analytes to improve detection of compounds at low concentrations. The steps
involved in the SPE are conditioning, sample loading, washing and elution (Figure 4.1).

During conditioning, SPE column is activated by methanol to open up hydrocarbon chains and to increases the surface area available for interaction with the analyte(s). In addition, it removes residues and contaminants from the manufacturing process and packing material. The column is then equilibrated with acidified deionized water and samples are loaded to bind to the sorbent while most of the sample matrix goes to the waste. Optimized flow rate and type of sorbent used is important for the interaction between analytes of interest and sorbent. The column is washed with a suitable solvent to remove the matrix that might interfere with the analytical column. The samples are then eluted with a suitable solvent which should be compatible with the mobile phase in the LC system. It is feasible to use large eluent volumes and stronger solvents to improve recovery. The resultant extract is evaporated and diluted with the aqueous LC eluent to a final volume 1ml. All the steps are dependent on the flow rate, solvent strength, pH, type of sorbent and physical and chemical properties of the compound.

4.1.2 LC-MS/MS

Mass spectrometer characterizes molecules on the basis of their mass-to-charge ratio ($m/z$). All mass spectrometers have the same basic components: a sample introduction system, an ion source, a mass analyzer that separates ions based on their $m/z$, a detector, a data collection computer and data analysis software.

LC-MS is a hyphenated technique, in which samples are first separated using liquid chromatography (LC), and then separated into the mass spectrometry. Parameters that may affect the LC separation are hydrophobicity and size of molecules, presence of specific functional groups, choice of stationary phase and their particle size, dimensions of the chromatographic column, flow rate, mobile phase composition, temperature and the compatibility of the mobile phase with the conditions required for efficient ionization of the analyte in the ion source of the mass spectrometer. Chromatography resolves individual components of the samples in time, while mass spectrometry selectively detects these components.

In the ion source, the first step is to evaporate the mobile phase and to ionize the compound of interest, thus it is essential to have volatile components in the mobile phase. A common and popular soft ionization technique which produces limited fragments is electrospray ionization (ESI).
A quadrupole mass spectrometer consists of four parallel rods located in a vacuum chamber. Each of the two pair of rods located opposite to each other is applied with DC and RF voltages, creating an electric field in the space between the rods where ion separation takes place. A combination of different DC and the RF voltages and RF frequency allows only ions with certain \( m/z \) to have stable trajectory while flying through the quadrupole while the rest of the ions collide with the rods. Advantages of the quadrupole mass analyzers are efficient ion transmission, high sensitivity of detection and the possibility to couple multiple quadrupoles to one another or to other types of mass analyzers.

The chemical analytical methods applied in this thesis utilize detection with triple quadrupole (or tandem) mass spectrometry (MS/MS). It has three components: two quadrupoles with a collision cell placed in between. The first mass analyzer selects ions of a specific \( m/z \) (parent ion), which then are fragmented in the collision cell by neutral gas molecules and the generated fragments (product ions) are directed into the second mass analyzer where they are resolved based on their \( m/z \) ratio and then detected. The retention time, the parent ion and mass-to-charge ratios of product ions are the basis of high degree of analytical specificity of LC-MS/MS as a technique. If both mass analyzers are fixed on transmission of the compound-specific parent and product ion, the mode is known as multiple reactions monitoring (MRM). It is not only highly specific but sensitive as it excludes all other ions except the targeted ones and is used in quantitative targeted analysis. All the chemical methods in the thesis (Papers I, II, IV and V) are in MRM data acquisition mode.

4.1.3 Off-line SPE/LC-MS-MS

The offline method was used in Paper II. The extract was injected into the LC system which passed through the guard column followed by analytical column. The target antibiotics were eluted with the mobile phase using a linear gradient elution method and were finally detected with the quadrupole mass spectrometer. The method was validated in terms of linearity, absolute and relative recoveries and inter- and intra-day precision.

4.1.4 On-line SPE/LC-MS-MS

Compared to the off-line method, an on-line SPE method is rapid, equally precise and efficient as it eliminates time-consuming evaporation and reconstitution steps and minimizes the need for sample handling. It has the obvious advantages of high sample throughput, minimal solvent utilization, low sample preparation times and small sample volumes. In this thesis 1 ml of water sample was preconcentrated on online SPE column coupled to an
LC instrument via a column-switching system and was used in Paper I, IV and V.

4.2 Quantification

The quantification in this thesis is done with internal calibration method based on isotopically-labeled internal standards (ISs). Signal intensities observed were related to the concentration of the analytes and in order to normalize the signal intensity, the same amounts of ISs were added to the calibration standards and the samples. The ratios of peak areas in the chromatogram for the ISs to the corresponding analyte(s) were used to calculate the concentrations in the samples. The positive identification of the analytes was based on similarity of the retention times of analytes in the samples and the calibration standards, and the selection of the appropriate precursor and daughter ions.

4.3 Biological analysis

4.3.1 Denaturing gradient gel electrophoresis (DGGE)

DGGE is used to assess and monitor the dynamics of diversity of microbial communities. The genes encoding the 16S rRNA are amplified and the fragments are separated in the polyacrylamide gel. DGGE exploits the fact that DNA molecules differing even by one nucleotide though of same length can be separated by electrophoresis while passing through a linear gradient of increasing chemical denaturants of urea and formamide. The DNA strands are separated or melted at specific temperature based on the hydrogen bonding between complementary base pairs and bases on the same strand thus their mobility is retarded when run on a polyacrylamide gel.

The advantage of the technique is that it provides rapid identification of the predominant species and may provide taxonomic information by excising, re-amplifying and sequencing specific DNA fragments.

4.3.2 Polymerase chain reaction (PCR) and Real-time PCR (RT-PCR)

Mikael Kubista et al. 2006 [64] has comprehensively reviewed the significance of PCR and RT-PCR. PCR is a rapid and sensitive method for the detection of resistance genes, especially for slow-growing and uncultivable microorganisms. It is particularly advantageous as it is estimated that >99% of the bacteria are uncultivable. Real-time PCR, or shortly written qPCR (quantitative PCR), quantitates DNA accumulation during the PCR cycling by adding a fluorescent molecule. The number of cycles it takes for the signal from different samples to reach a certain
fluorescence threshold is called Cycle of threshold, $C_t$. It is assumed that when the samples reach this level they contain the same number of DNA molecules. If samples with known concentrations are run along with unknown samples, a standard curve can be plotted as $C_t$ versus DNA concentration to quantify the unknown samples. RT-PCR has a large dynamic range, more than eight orders of magnitude. A difference in one cycle means a two times difference in initial DNA amount provided the reaction proceeds with 100% efficiency.

A large variety of different non-specific DNA binding dyes (SYBR® green, Ethidium bromide) and specific hybridization probes (TaqMan™) that can anneal in a position “between” the primers and labeled primers (LUX™) are available.

Number of studies has demonstrated RT-PCR are fast, reliable and sensitive methods for the detection and quantification of ARGs and qPCR (RT-PCR) is used in Paper II, III and IV.
5. Antibiotics effects on biodiversity and resistance development

5.1 Constructed wetlands

Constructed wetlands (CWs) are shallow man-made water bodies that support a large growth of fauna and flora and facilitate the removal of pharmaceuticals via natural processes involving plants, microorganisms, solid matrix components and sunlight [65-67]. Recently, it has been shown that passing waste water streams through CWs can serve as a cost-effective and promising alternative to conventional tertiary treatment methods for removing and reducing levels of nitrogen, phosphorous, pharmaceuticals, antibiotics and the BOD [6, 68, 69].

The bacterial communities in CWs are involved in water purification in wastewater effluents through processes such as ammonia oxidation, nitrogen fixation and denitrification. Therefore high concentration of antibiotics in the influx may affect the bacterial community composition.

5.1.1 CWs in Plönninge

The study was performed at pilot-scale wetlands in Plönninge [70] near Halmstad in the south-west of Sweden. They were constructed in 2002 and consisted of 18 similar wetland basins located within an experimental area of 76 x 32 m. Each wetland covered a rectangular area of 40m² (10 x 4 m) at ground level and were placed with a distance of 4 m from each other. For detail experimental conditions the readers are referred to Paper II.

5.2 Effects on bacterial biodiversity

The diversity of the bacteria in the environment is high and based on the differing nutrient needs of the microorganisms and the different environmental compartments available. Higher biodiversity of the same species or different species bacteria may increase the adaptation and thus decrease the adverse effects on the total population. It is assumed that the high biodiversity of microorganisms is one of the reasons of their high elasticity and resilience with respect to their functions such as biodegradation of organic compounds as well as their reactions to toxic effects. Furthermore, the adaptation period is shorter if the total diversity is high because there is strong probability that fast adapting organisms or
organisms specialized in a certain compound are present. Some bacteria also utilize the biodegradation products from other groups of bacteria.

**In Paper II,** CWs were spiked continuously with nine antibiotics at environmentally relevant concentrations for 25 days and no significant differences in bacterial diversity and evenness at the family level was observed. On comparison of days; Day 220 and 460 had significantly lower ($P < 0.05$) shannon indices $H'$ values than to Day 50, which had the highest mean value of all days. Furthermore F (unspiked wetland) had the highest mean value of $H'$ which suggests that it had greater bacterial diversity than D (spiked wetland) (Figure 5.1).

![Figure 5.1](image-url)

**Figure 5.1.** The Shannon Index ($H'$) was compared between the antibiotic spiked wetlands A–D and control wetlands E–H (panel I). The diversity was significantly higher ($P < 0.05$) in the control wetland F than in the antibiotic spiked wetland D. However, no other significant differences could be seen between the other wetlands. When comparing the evenness ($J'$) between the different wetlands (panel II), control wetland G displayed a significantly ($P < 0.05$) higher evenness than control wetland E. No other statistically significant differences could be seen.

The results in **Paper II** suggests that either the spiked environmentally relevant concentrations are low to impact the diversity of the bacterial community or perhaps some factor, other than antibiotics concentration is responsible for diversity. The impact of high concentration is also demonstrated by Weber et al. [71] in his study to assess the bacterial community species distribution and catabolic capabilities. Exposure to 10000 times higher concentration of ciprofloxacin than the study in Paper II for a period of 5 days adversely affected the activity and catabolic activities but functionality returned to normal after 2-5 weeks [71].
5.3 Effects on development of ARGs

In Paper III, it was investigated whether antibiotic exposure in Paper II influenced the proliferation and dissemination of ARGs. Vancomycin resistance gene (vanB), quinolone resistance gene (qnrS), sulphonamide resistance gene (sulI), tetracycline resistance genes (tetA and tetB), macrolide, lincosamide and streptogramin resistance gene (ermB), trimethoprim resistance gene (dfr1) and the gene coding for the integrase on class 1 integrons (intI1) were determined as presented in table 5.1. Apart from qnrS all other genes either were detected or were quantifiable at varying concentrations.

Table 5.1. Detection, quantification and levels of ARGs in Paper III.

<table>
<thead>
<tr>
<th>ARGs</th>
<th>Detected (samples)</th>
<th>Quantifiable (samples)</th>
<th>Levels (gene copies / 10^6 16S rDNA copies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van B</td>
<td>DNA 12 out of 72</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>RNA 9 out of 69</td>
<td>non</td>
<td></td>
</tr>
<tr>
<td>qnr S</td>
<td>DNA Non</td>
<td>non</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNA Non</td>
<td>non</td>
<td></td>
</tr>
<tr>
<td>SulI</td>
<td>DNA All</td>
<td>all</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>RNA All</td>
<td>62 out of 69</td>
<td></td>
</tr>
<tr>
<td>tet A</td>
<td>DNA 54 out of 72</td>
<td>29 out of 72</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>RNA 4 out of 69</td>
<td>non</td>
<td></td>
</tr>
<tr>
<td>tet B</td>
<td>DNA 48 out of 72</td>
<td>5 out of 72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNA 4 out of 69</td>
<td>non</td>
<td></td>
</tr>
<tr>
<td>ermB</td>
<td>DNA 7 samples</td>
<td>4 samples</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>RNA 1 samples</td>
<td>non</td>
<td></td>
</tr>
<tr>
<td>dfr1</td>
<td>DNA 7</td>
<td>Non</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNA 4</td>
<td>non</td>
<td></td>
</tr>
<tr>
<td>IntI1</td>
<td>DNA all</td>
<td>all</td>
<td>4900</td>
</tr>
<tr>
<td></td>
<td>RNA all</td>
<td>all</td>
<td>440</td>
</tr>
</tbody>
</table>

No significant differences in concentrations or prevalence of ARGs could be observed between antibiotic treated and control wetlands. The results suggested that the antibiotics spiked in environmentally-relevant concentrations have no effect on either dissemination of ARGs or resistance development of the bacterial community. Furthermore RNA gene concentrations were relatively lower than the DNA gene which suggests that these genes were rarely expressed. In addition to this, no significant difference between spiked and control wetlands was observed for Class 1 integrons, thus suggesting that integrons concentration is not affected by antibiotics.
Gullberg et al., 2011 [72] showed that antibiotic concentrations below MIC-values can select for resistant bacteria *in vitro* in considerably less complicated microbial communities and at antibiotic concentrations about 1000 times higher than in Paper III. It suggests that wetlands are more complex ecological systems and resilient to the resistance development effects of low levels of antibiotics commonly encountered in wastewater as used in Paper III. Nevertheless 25 days exposure in CWs is a small duration and wetlands for purification of water are obviously exposed for long duration of time to low level of antibiotics, hence, further studies for long exposure time are needed to assess the effects.
6. Monitoring anti-infectives and ARGs – national perspectives

6.1 Pakistan: Background and sampling sites

Pakistan is a semi-industrialized economy with agriculture providing 45% of the employment. Rivers, and in particular River Indus, plays a key role in providing water resources for agriculture and is also used for energy generation. In paper IV, 19 sampling sites were selected on six rivers, one dam, one canal, a sewage drain in the vicinity of three hospitals and four drug formulation facilities as shown in figure 6.1.

The chemical analysis method used was modified from the analytical method used in Paper I. Method used to detect / quantify ARGs were identical to the one used in Paper III.

6.2. Antibiotics in rivers, canal and sewage drain nearby hospitals

Results of the 6 most commonly found antibiotics are summarized in Figure 6.2, focusing on the levels found in ng L$^{-1}$ in River Ravi, Lahore Branch Canal and the sewage drain nearby three major hospitals. A clear increasing trend in the levels of antibiotics was seen in the downstream River Ravi (R3) as compared to upstream (R1). Results clearly show the impact of the municipal sewage waste from the 10 million city, Lahore. Highest concentrations measured were 2700 ng L$^{-1}$ and 1700 ng L$^{-1}$ for SULM and TRI respectively. A slight increase in antibiotic concentrations could also be seen in the canals in the city center, when comparing the upstream (C1) and downstream canal (C2) samples. However, the highest levels were observed in the sewage drain nearby three hospitals, concentrations found were 4600 ng L$^{-1}$, 3200 ng L$^{-1}$, 2200 ng L$^{-1}$ for SULM, OXY and TRI respectively suggesting possible contribution from nearby hospitals and household waste. The levels found at other sites (R4, R5, R6, R7, R8, R9, R10 and D) were in almost all cases in low ng L$^{-1}$ with a few exceptions.
Figure 6.1. Map of the region with sampling sites indicated. R1–R10 are rivers, D is the Rawal dam, C1–C2 is the Lahore branch canal, SD is the sewage drain nearby hospitals and P1–P4 are the pharmaceutical formulation facilities.
Figure 6.2 Average concentrations (n=3) of antibiotics in River Ravi, canal and sewage drainage nearby hospitals.

6.3. Antibiotics nearby drug formulation facilities

The top 6 antibiotics in terms of concentrations found nearby DFF are shown in Figure 6.3. Levels found at sites, P1a, P1b and P2 were comparable to levels found at sites C1 and C2, and levels found at P3; 4700 ng L\(^{-1}\) and 1000 ng L\(^{-1}\) for SULM and TRI respectively, were comparable to the levels found in the sewage drain nearby three hospitals (SD). Furthermore, the highest concentrations were observed at site P4 with the top 6 antibiotics ranging from 6200 ng L\(^{-1}\) to 49000 ng L\(^{-1}\) for CIP and SULM respectively. Levels measured at site P4 were the highest in this study and emphasizes the DFFs as significant point sources. Larsson et al. 2007 and Fick et al. 2009 [3, 25] have found drug production facilities as being the single largest contributing point source of antibiotics to the environment as referred earlier in chapter 3.
**Figure 6.3** Average concentrations (n=3) of antibiotics nearby DFF.

### 6.4. Antibiotic resistance genes in rivers, canal and nearby DFF

Levels of ARGs are low in most of the sites as shown in Figure 6.4. However increasing levels of *sulI* and *dfr1* are seen in downstream River Ravi (R3) as compared to upstream (R1) and near city R2 which suggest possible contribution of ARGs by the municipal sewage waste water caused by either an increased selection at higher antibiotic concentrations or a contribution of ARGs directly from the waste water. On the basis of increasing levels of antibiotics, in particular SULM and TRI found in downstream water samples (section 6.1.1) and soil samples (Paper IV), one can speculate regarding correlation of higher *sulI* and *dfr1* levels with higher downstream antibiotic concentrations. Highest ARG levels were detected at the site with the highest antibiotic levels (P4). This site showed a direct correlation between the levels of antibiotics found and the presence of ARGs.

Of high concern is that integrase *intI1* could be detected at almost all the sites and that the site R3 (downstream) had higher levels than R1 (upstream) and that the highest levels were observed in samples at P4.
Figure 6.4 Concentrations of antibiotic resistance genes.

6.5 Sewage epidemiology surveillance

Sewage epidemiology is a new approach based on the fact that drugs are metabolized in the body and the metabolites along with parent drugs are excreted in the urine and end up in the influent wastewater. Parent drug and its metabolites can be measured in the influent wastewater and then back-calculated to assess their consumption in communities and populations in a rather direct, quick and objective way. However several factors such as stability of the analyte(s) during transport to the WWTP, the percentage of drug metabolized in the body, the flow rate of the wastewater stream and the number of inhabitants served by the WWTP should be taken into account for accurate back-calculations. The approach has already been used in number of studies to access the usage of illicit drugs in the communities [73, 74].

In Paper V the sewage epidemiology approach was used to estimate the oseltamivir carboxylate (OC), an active metabolite of the drug oseltamivir in wastewater to examine the compliance of the population in the WWTP catchment area during the peak of the second wave of the influenza pandemic in southern England.
OC was measured from twenty four time proportional hourly influent samples from two WWTP catchments with a population of ~6000 and ~200000 people in Benson and Oxford respectively in southern Oxfordshire, England, see figure 6.5. Measured OC was compared with two complementary sources of national government statistics to assess compliance rates.

Figure 6.5. Catchment area of River Thames, England.

The projected concentration of OC in ng L$^{-1}$ was calculated using the equation $((D_p \times D_M)/(P \times L)) \times 10^9$, where $D_p$ is the population predicted to consume oseltamivir, $D_M$ is the mass equivalent of OC consumed per day and is 0.15 g/d, the defined daily dose for oseltamivir; $P$ is the population in the catchment area of each WWTP and $L$ is the volume of wastewater per person (230 L).

A method based on on-line SPE coupled to LC-MS/Ms (Paper I) was used to measure the OC. The mean concentration of OC in Benson WWTP was 394 ng L$^{-1}$ with a maximum of 2070 ng L$^{-1}$ and minimum of 59 ng L$^{-1}$ and the mean concentration in Oxford WWTP was 350 ng L$^{-1}$ with a maximum of 550 ng L$^{-1}$ and minimum of 257 ng L$^{-1}$. The average load of 24 hour period for both the WWTPs is given in figure 6.6.
Figure 6.6. Calculated hourly influent load of OC (mg/h) for Oxford WWTP (closed) and Benson WWTP (open).

Based on these measurements the total usage was calculated and this ranged from 3-4 people in Benson and 120-154 for Oxford. These calculated values were consistent with the Government statistics and suggests that that compliance of the drug was 45-60% in the region.
7. Conclusions and future perspectives

In monitoring programs, trace contaminants are often enriched by solid phase extraction (SPE) prior to analysis which is a labor-intensive and time-consuming, particularly when dealing with high volumes. This thesis has demonstrated the importance of the development of rapid, selective and sensitive online methods combining solid phase extraction (SPE) of high volume samples with liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the simultaneous analysis of anti-infectives.

CWs can serve as a useful alternative to conventional tertiary treatment methods for removing organic pollutants via natural processes involving plants, microorganisms, solid matrix components and sunlight. It was illustrated that the bacterial community composition in pilot-scale wetlands showed no significant differences when exposed to nine antibiotics at environmentally relevant concentrations. Furthermore, it was also demonstrated that there was no significant differences in levels of ARGs between antibiotic treated and control wetlands, thus indicating that such levels do not promote resistance development in pilot-scale constructed wetlands.

Antibiotics release from hospitals, WWTPs, inappropriate disposal, veterinary and agriculture usage into the natural ecosystems serve as a breeding ground for the development of resistance due to natural selection and further acts as reservoirs for propagation and proliferation of ARGs. The thesis demonstrates the possible correlation of higher antibiotic concentrations with the higher levels of ARGs found at contaminant sites in Pakistan. It also shows the impact of a big city like Lahore on downstream higher levels of antibiotics and ARGs.

Sewage epidemiology surveillance is a new approach where parent drug and its metabolites are measured in the influent wastewater and then back-calculations are made to assess the consumption of drugs in communities and populations. In the thesis, OC was measured in twenty four time proportional hourly influent samples from two WWTPs in south England. The compliance of the drug was evaluated and on comparison found to be consistent with government statistics.
Future aspects

Numbers of studies have been carried out to determine the sources and occurrence of pharmaceuticals including antibiotics in various natural environments but still data on fate and effects is limited for risk assessment and management. Furthermore little is known about the metabolites and their activity.

In future studies on fate of metabolites and transformed products is of great significance. An important question in this regard needs to be addressed is whether metabolites such as glucuronides, methylates, glycinates, acetylates and sulfates are still active and whether they can be biodegraded by bacteria during sewage treatment or in the environment.

With reference to ARGs selection and spread, an important fact still to be determined is whether selective pressure is more significant or the already resistant bacteria discharged in the waste water.

There is also need to comprehensively tackle the problem of drug emissions and this could be achieved by development of drugs that are equally efficacious and degradable, promoting rational drug prescription and usage in humans, animals and agriculture, disposal control and technical treatment especially for potentially harmful and persistent drugs.
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References


