Environmental pollution from pharmaceutical manufacturing

-effects on vertebrates and bacterial communities

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ABSTRACT

High levels of pharmaceuticals, including fluoroquinolones (FQs), have been detected in the effluent from an Indian waste water treatment plant serving bulk drug production intended for the global market. Responses from short-term effluent exposure were studied in fish and rats through explorative analyses of hepatic mRNA abundance, enzyme activities and blood chemistry parameters. Exposure of rainbow trout to 0.2% effluent for five days altered hepatic gene expression and increased Cyp1a activity as well as blood plasma phosphate and cholesterol. In contrast, no effects could be demonstrated in rats tube-fed with effluent. Thus, exposure to effluent from drug manufacturing affects aquatic wildlife. No toxic effects were observed in rats after short-term exposure but risks associated with higher doses of effluent or a longer exposure time cannot be excluded.

High concentrations of FQs were found in sediment from the Indian river receiving drug-contaminated effluent, while no FQs were detected in sediment sampled near a municipal Swedish waste water treatment plant. Metagenome sequencing showed that resistance genes for several classes of antibiotics as well as genetic mobility elements were enriched in Indian sediments compared to Swedish samples. Selected antibiotic resistance genes were studied with qPCR in well water and soil from Indian villages. FQs were detected in samples from villages located <3 km from waterways with documented drug contamination. No enrichment of quinolone resistance genes (*qnr)* were seen in FQ-contaminated well water or soil while differences over seasons were observed for *sul2*, a sulfonamide resistance gene, and *intI1*, a class 1 integrase. Also, *qnr* were analyzed in human fecal samples. Three *qnr* genes were prevalent in fecal samples from Indians living in FQ-polluted as well as in FQ-free villages. The same three genes were detected, but less commonly, in stool samples from a group of Swedish students.

In conclusion, these studies demonstrate that discharges from antibiotic production lead to promotion of resistance genes and mechanisms facilitating their mobility in highly contaminated aquatic environments. Additional studies are required to elucidate the consequences of lower antibiotic concentrations in well water and soil, and the risk for transfer of antibiotic resistance genes from environmental bacteria to human intestinal flora. Once established, antibiotic resistance can rapidly spread over an extensive geographical area. Despite current knowledge gaps, the toxicity to wildlife and potential detrimental consequences for human health call for immediate and collaborative actions to improve waste management from drug manufacturing.

Svensk sammanfattning

Läkemedelssubstanser är designade (eller utvalda) för att kunna påverka biologiska system och processer genom att binda till olika målstrukturer i våra kroppar. Många andra arter har dock målmolekyler som liknar våra vilket innebär att andra organismer kan påverkas om läkemedel kommer ut i miljön. De senaste åren har det publicerats flera artiklar som visar att höga halter av läkemedel släpps ut i miljön vid tillverkningen av läkemedelssubstanser. I det utgående avloppsvattnet från ett reningsverk i Patancheru, Indien som renar vatten från läkemedelsproduktion, hittades mycket höga koncentrationer av flera olika sorters läkemedel, framförallt fluorokinolon-antibiotika.

I studierna som ingår i den här avhandlingen har vi undersökt vilka effekter exponering av det indiska avloppsvattnet har på två ryggradsdjur samt hur bakterier i olika indiska miljöer påverkats av utsläppen av antibiotika. I den första studien lät vi regnbågslax simma i avloppsvatten som var utspätt 500 gånger under fem dagar och analyserade sedan fiskarnas blod samt undersökte hur genuttrycket i levern påverkats. Både genuttrycket och aktiviteten av Cyp1a, ett enzym som bland annat är involverat i att bryta ner främmande ämnen, var förhöjda hos fisk som exponerats för avloppsvattnet. I nästa försök sondmatades råttor med avloppsvatten men här kunde vi inte se några akuta effekter på varken genuttryck eller blod-parametrar.

Avloppsvattnet från det indiska reningsverket släpps ut i en närbelägen flod och i sediment därifrån hittade vi mycket höga koncentrationer av fluorokinoloner. För att undersöka hur detta påverkar bakterier analyserade vi DNA i indiska sedimentprover och jämförde det med prover som samlats in nära ett svenskt reningsverk där vi inte hittade någon antibiotika i sedimenten. Vi fann höga nivåer av bland annat kinolon-resistensgener (*qnr*) i de indiska proven medan dessa gener inte hittades i de svenska proven. Genom att undersöka ett mycket stort antal slumpmässiga DNA sekvenser i ett prov kan man i princip leta efter alla hittills beskrivna gener samtidigt. Däremot är den sekvenseringsmetod vi använde inte så känslig, vilket innebär att ovanliga gener kan vara svåra att upptäcka. Därför undersökte vi också *qnr*-gener i flodsedimenten med hjälp av en mycket känsligare analys, kvantitativ PCR. Med denna metod kunde vi identifiera

ytterligare *qnr*-gener i sediment kring det indiska reningsverket medan ytterst få hittades i svenska prover. Vid sekvenseringsanalysen upptäcktes också höga nivåer av *sul2*, en gen som ger resistens mot sulfonamid-antibiotika i de indiska flodsedimenten tagna nedströms om reningsverket. Dessutom hittades många kopior av genen *intI1* som förknippas med klass 1 integroner, genetiska element som kan samla på sig flera resistensgener på rad. Resistensgenerna kan dessutom klippas ut och klistras in i andra integroner med hjälp av *intI* och höga nivåer av denna gen kan därmed innebära en ökad risk för att resistensgener sprids mellan bakterier.

I nästa studie tog vi prover på brunnsvatten och jord i området kring Patancheru för att undersöka om fluorokinoloner förorenat också dessa miljöer. Vi fann att prover från byar som låg upp till3 km från förorenade vattendrag innehöll fluorokinoloner även om koncentrationerna var betydligt lägre än i avloppsvatten och flodsediment. Kvantitativ PCR användes för att analysera *qnr*, *sul2* och *intI1* i brunnsvatten och jord men vi kunde inte hitta stöd för hypotesen att *qnr*-gener var överrepresenterade i prover från fluorokinolon-kontaminerade byar. För *sul2* och *intI1* fanns tendenser till ansamling i byar med fluorokinolon-förorening men resultaten varierade mellan olika provtagningsomgångar varför resultaten ska tolkas försiktigt.

Till sist undersöktes också den potentiella länken mellan miljöförorening av fluorokinoloner och *qnr* i humana avföringsprover från invånare i kinolon-kontaminerade och kinolon-fria indiska byar. Förekomsten av *qnr* jämfördes också med avföringsprover från en grupp svenska studenter. Kinolon-resistensgenerna *qnrB*, *qnrD* och *qnrS* var vanliga i de indiska avföringsproverna oavsett om personen bodde i en kinolon-kontaminerad eller kinolon-fri by. Samma tre gener hittades också i de svenska avföringsproven, men mycket mer sällan.

Sammanfattningsvis tyder de uppenbara effekterna på fisk, trots den höga utspädning som användes i försöket, på att avloppsvatten från indisk läkemedelsindustri kan påverka vattenlevande organismer inom ett stort geografiskt område. Risken för akuta, toxiska effekter på landlevande djur verkar vara små även om kroniska effekter associerade med längre exponeringstid eller

högre doser av avloppsvatten inte kan uteslutas. Resultaten tyder också på att utsläpp från läkemedelstillverkning kan ha betydelse när det gäller utveckling och spridning av antibiotikaresistens i vattenmiljöer med höga halter antibiotika. Ytterligare studier behövs för att klarlägga konsekvenserna av de lägre antibiotikakoncentrationerna som hittades i brunnsvatten och jord, samt för att utvärdera risken för att resistensgener från miljöbakterier sprids till human tarmflora.

Trots att det fortfarande finns betydande kunskapsluckor när det gäller mekanismerna bakom utveckling och spridning av antibiotikaresistens kan riskerna med underdimensionerade, eller frånvaro av, åtgärder bedömas tillräckligt allvarliga för att omedelbara insatser ändå ska vara motiverade enligt försiktighetsprincipen. Eftersom antibiotikaresistens snabbt kan sprida sig över världen spelar det mindre roll var den först uppkommer. Internationella överenskommelser och gemensamma strategier för att minimera utsläpp från läkemedelstillverkning är sannolikt nödvändiga för att åstadkomma bestående effekter i ett globalt perspektiv.

List of papers

This thesis is based on the following articles and manuscripts:

Paper 1 **Pharmaceutical industry effluent diluted 1:500 affects global gene expression, cytochrome P450 1A activity, and plasma phosphate in fish**
Gunnarsson L, Kristiansson E, Rutgersson C, Sturve J, Fick J, Förlin L, Larsson DGJ.
Environ Tox Chem, 2009, 28: 2639–2647

Paper 2 **Oral exposure to industrial effluent with exceptionally high levels of drugs does not indicate acute toxic effects in rats**
Rutgersson C, Gunnarsson L, Fick J, Kristiansson E, Larsson DGJ.
Environ Tox Chem, 2013, 32: 577–584

Paper 3 **Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements**
Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegård B, Söderström H, Larsson DGJ.
PLoS One, 2011, 6(2): e17038

Paper 4 **Antibiotics and antibiotic resistance genes in Indian well water and soil contaminated by industrial pollution**
Rutgersson C, Fick J, Marathe N, Kristiansson E, Janzon A, Flach C-F, Larsson DGJ.
Manuscript

Paper 5 **Quinolone resistance (qnr) genes in the gut flora of people living in an antibiotic-contaminated environment**
Rutgersson C, Marathe N, Angelin M, Kristiansson E, Moore ERB, Shouche Y, Johansson A, Flach C-F, Larsson DGJ.
Manuscript

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CONTENTS

ABSTRACT 3
SVENSK SAMMANFATTNING 4
LIST OF PAPERS 7
CONTENTS 8
INTRODUCTION 10
Environmental effects from pharmaceuticals 11
Sources of pharmaceutical pollution 13
Antibiotics 15
Antibiotic resistance 16
Horizontal gene transfer 18
The environmental resistome 19
Quinolone antibiotics and quinolone resistance 20
AIMS 24
Specific aims 24
METHODOLOGICAL CONSIDERATIONS 25
Environmental sampling 25
Animal models 26
Human sampling 29
Chemical measurements 30
Analysis of genomic DNA and mRNA expression 30
Microarrays 30
Quantitative polymerase chain reaction 32
Metagenome sequencing 34
Enzymatic assays 34
RESULTS AND DISCUSSION 36
Pharmaceutical contamination in Patancheru 36

Direct effects **38**
Antibiotic resistance **44**
Metagenome sequencing 44
qPCR of resistance genes 45
Risks for development and spread of antibiotic resistance in contaminated environments 49
Findings of high environmental levels of pharmaceuticals **54**
FUTURE PERSPECTIVES 57
Proposed focus points for future research **57**
Improved management of discharges from drug production **58**
ACKNOWLEDGEMENTS 60
REFERENCES 62

Introduction

Today, it may be difficult for most of us to fully realize the immense impact that the era of discovery and large-scale production of pharmaceuticals during the past century have had on human survival and life quality. However, the nature of pharmaceutical substances is somewhat dual; besides their beneficial and sometimes essential qualities, this group of compounds possesses characteristics which have caused them to emerge as pollutants of concern if released into the environment.

In contrast to other molecules sometimes considered environmental pollutants, pharmaceuticals are actively selected or developed for their ability to evoke a biological response, *i.e.* interact with target molecules in the human body (or in microorganisms for antimicrobials). Often, they are derivatives of naturally occurring compounds, further chemically engineered for example to better withstand rapid metabolic breakdown to extend the treatment window. However, an improved pharmaceutical stability can lead to incomplete metabolism during passage through the human body. Hence, drugs (or metabolites thereof) with uninterrupted biological activity can in many cases be excreted in urine and faeces. Municipal sewage treatment plants (STPs) are generally not equipped with the technology required for complete degradation and removal of such a heterogeneous group of compounds as are pharmaceuticals. Thus, many of the more persistent drugs are able to pass the treatment processes and end up in the aquatic milieu. Since some decades back, active pharmaceutical ingredients (APIs) and metabolites from human medicines have been reported in environmental samples (for reviews, see Halling-Sørensen et al. 1998; Heberer 2002; Kümmerer 2008). Risks for undesirable effects on wildlife are evident when considering that many of the human drug targets are evolutionary conserved also in other organisms (Gunnarsson et al. 2008). Furthermore, to reduce the risk for unwanted side effects in humans during treatment, pharmaceuticals are generally designed to induce precise and well-defined responses already at low concentrations. As a consequence, many drug compounds may be potent enough to affect wildlife over large areas, far from actual discharge sites.

Environmental effects from pharmaceuticals

Many pharmaceutical substances released into the environment eventually end up in waterways. As a consequence, water-living organisms, particularly those that ventilate water, may be at risk due to continuous substance exposure. In the early 90's, Purdom et al. (1994) observed fish with intersex characteristics near sewage treatment plants in England. Later, natural estrogens and ethinylestradiol, the synthetic estrogen commonly used in contraceptives, were found in effluents from British sewage treatment plants (Desbrow et al. 1998). Several studies have confirmed that estrogens, particularly ethinylestradiol, can impair reproduction in fish (Larsson et al. 1999; Jobling et al. 2002; Parrott and Blunt 2005; Kidd et al. 2007).

There are also reports of how pharmaceutical pollution can have devastating consequences for terrestrial vertebrates. Diclofenac, a non-steroid anti-inflammatory drug, was routinely used for treatment of domestic livestock in India and Pakistan. Deceased cattle are often left out in the open, available for *e.g.* scavenging birds. The amount of diclofenac residues in tissues of treated farm animals was enough to cause acute and fatal renal failure and a nearly complete extinction of several vulture species of the *Gyps* genus on the Indian subcontinent (Oaks et al. 2004; Shultz et al. 2004).

Due to the generally complex combination of drug compounds and other chemical pollutants in nature it is often a laborious task to establish clear causal links between specific environmental drug contamination and adverse effects on wildlife. Nonetheless, numerous exposure experiments with different types of drugs have been performed in the more easily controlled laboratory setting. For example, exposure studies have suggested that environmentally relevant concentrations of levonorgestrel and oxazepam affect fish reproduction and behavior, respectively (Zeilinger et al. 2009; Brodin et al. 2013). Albeit sometimes oversimplified in comparison to the intricate processes that might take place in nature, this type of exposure studies may be of high importance when it comes to increasing the knowledge regarding effects from environmental pollution and to identify substances of particular environmental concern. Valuable tools in the assessment of susceptibility, exposure and effects on wildlife increasingly being

used in both laboratory and field studies are biomarkers; molecules or activities indicating a biological state. Alterations in concentration or activity rate of the corresponding biomarker can aid in identifying the mode of action for the observed toxicity and offer hints to what substances are most harmful in a complex sample mixture. For example, in juvenile and male fish the abnormal gene induction of vitellogenin, an egg yolk precursor protein typically produced by female oviparous organisms, is commonly used for assessing exposure to endocrine disruptive compounds (Jobling et al. 2002; Larsson et al. 1999; Purdom et al. 1994; Routledge et al. 1998; Sumpter and Jobling 1995). Another common biomarker of exposure in fish is the cytochrome P450 family (Cyp) enzymes which are involved in the biotransformation of toxicants, including pharmaceuticals, aiming to increase their water solubility and facilitate excretion. The *Cyp1a* gene is induced by a range of xenobiotics including aromatic oil hydrocarbons and dioxins and the activity of the enzyme can be both promoted and inhibited by pharmaceuticals (Hu et al. 2007). Also, the Cyp1a activity can lead to the formation of prooxidants which in turn can cause a condition referred to as oxidative stress and potential damage on both proteins and lipids if not restored by the antioxidant defense mechanisms (Carney Almroth 2008).

There are numerous criteria defining an excellent biomarker including robustness between measuring techniques, sufficient specificity and sensitivity to enable identification of individual or groups of toxicants at environmentally relevant concentrations. Ideally, there should also be a correlation between the degree of exposure and biomarker response. Commonly, a single biomarker does not fulfill all these requirements why a set of markers is often used in combination and the identification of new and reliable biomarkers is a continuous scientific pursuit.

Sources of pharmaceutical pollution

Emissions originating from human use and excretion (including individual households and hospitals, retirement homes and other health care institutions) are generally considered the main source for pharmaceutical pollutants (but environmental drug residues can also originate from inapt disposal of unused medicines. Also, leachates from landfills of pharmaceutical waste can pollute the surrounding environment (Holm et al. 1995). Additionally, during sewage treatment, drug compounds can adsorb to solid particles in sludge (Kümmerer 2009). As sludge can be rich in organic nutrients it is sometimes used as fertilizer for agriculture why drug residues can be transferred to topsoil. Also, manure from farm animals treated with pharmaceuticals contains incompletely metabolized active ingredients which may eventually reach waterways, *e.g.* through run-offs after precipitation.

A wide range of pharmaceutical substances have been detected in the environment in different parts of the world and as measuring techniques keep improving and the risks associated with environmental drug contamination are given more attention, this number will likely continue growing. In treated sewage water and recipient waterways drug residues and metabolites are generally found in concentrations up to low µg/L. Drug residues have also been detected in drinking water, albeit only for a smaller set of compounds and generally at lower concentrations (ng/L) (Benotti et al. 2008; Mompelat et al. 2009). Accordingly, any direct risks for human health are considered low (Touraud et al. 2011). The contribution of pharmaceutical pollution from the *production* of drugs has until recently been assumed negligible, to some extent likely due to that the assumed great value of active substances would in itself prevent any major discharges (Williams 2005). Pharmaceutical production as a source for environmental contamination was proposed in a review from 1998 (Halling-Sørensen et al. 1998) but received little attention both in the scientific community and the public society. However, during the past few years, releases from several pharmaceutical industries have been documented as significant sources of local point contamination (Figure 1).

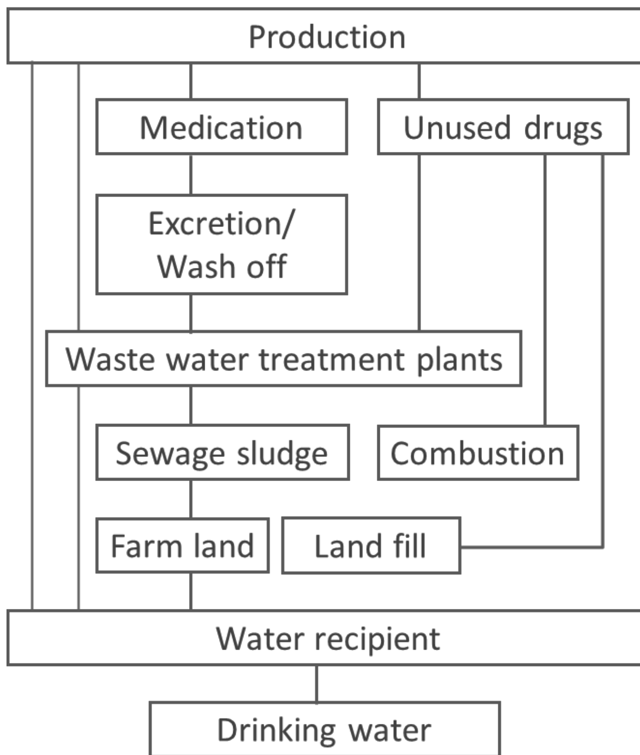

Figure 1. Schematic showing the main routes for pharmaceuticals to the environment. The environmental pollution of active pharmaceutical ingredients from the production of pharmaceuticals has been the focus point of the present thesis.

In Patancheru, near the massively industrialized city of Hyderabad, India, our group demonstrated very high levels of several pharmaceuticals in the final effluent from a plant treating process water from over 90 bulk drug industries (Larsson et al. 2007). The effluent was a complex mixture of substances; *e.g.* antihypertensives, histamine receptor antagonists and antidepressants, but first and foremost it contained unprecedented concentrations of broad-spectrum fluoroquinolone (FQ) antibiotics (Larsson et al. 2007). Eleven substances were detected in concentrations >100µg/L and ciprofloxacin, the most abundant compound in the treated water, was present at more than 30 mg/L (Table 1).

Table 1. The top 11 pharmaceuticals detected in effluent from a waste water treatment plant in India serving bulk drug industries (Larsson et al. 2007). The effluent was used in the fish and rat exposure experiments in paper 1 and paper 2 respectively.

Active ingredient	Type of drug	Concentration (µg/L)
Ciprofloxacin	Fluoroquinolone	28,000-31,000
Losartan	Antihypertensive	2,400-2,500
Ceterizine	Antihistamine (allergy)	1,300-1,400
Metoprolol	Antihypertensive	800-950
Enrofloxacin	Fluoroquinolone	780-900
Citalopram	SSRI	770-840
Norfloxacin	Fluoroquinolone	390-420
Enoxacin	Fluoroquinolone	150-300
Lomefloxacin	Fluoroquinolone	150-300
Ofloxacin	Fluoroquinolone	150-160
Ranitidin	Antihistamine (dyspepsia)	90-160

This was estimated to correspond to a daily release into the recipient river of about 44 kg, corresponding to 5 days' average consumption in Sweden. A few years later, 14 mg/L of ciprofloxacin was detected in the same effluent (Fick et al. 2009). Previous toxicity tests with the effluent from the Indian waste water plant had showed that even in highly diluted effluent had adverse effects on bacterial, plant and animal standard species *(Aliivibrio fischeri, Lactuca sativa* and *Daphnia magna,* respectively) (Larsson et al. 2007). However, due to the highly complex mixture of the effluent including pharmaceuticals, solvents and human sewage, the mechanisms behind the toxicity and the potential effects for aquatic vertebrates and terrestrial species were not yet elucidated.

Antibiotics

Also in the microbial world, there is a constant battle over space and resources. During evolution, microbes have developed techniques to attack or defend themselves against intruders. One of the weapons in this arsenal includes the

production and excretion of chemical substances that have negative effects on other microorganisms. In some cases, humans have learnt to use this to their advantage; by developing and adapting these compounds we have created many of the antibiotics taken for granted in modern health care.

The term "antibiotic" was used to describe a molecule produced by a microorganism that could inhibit the growth of other microorganisms (Waksman and Woodruff 1942). Since then, the definition has broadened and today also semi- or fully synthetic compounds are generally included in the term. Antibiotic substances are distinguished from other human pharmaceuticals in that they are selected due to their selective toxicity; potent effects on microbial systems while minimal influence on eukaryotic cells. In addition to treatment of already established infections, antibiotics are also of immense importance for prevention of infections in clinical settings where this would have detrimental consequences, *e.g.* during invasive surgery, in neonatal care units and for patients simultaneously treated with immunosuppressants as during transplantational surgery.

Comprehensive data on sales and use of antibiotic substances is scarce or missing from many countries. During 2010, Swedish pharmacies sold of over 55 million defined daily doses of antibiotics (http://www.apotekensservice.se). However, the majority of the antibiotics are intended for non-human use as they are heavily used prophylactically in aqua-, and agriculture, and for veterinary purposes and growth promotion in livestock farming (Cabello 2006; Stanton 2013).

Antibiotic resistance

In addition to adverse effects on local flora and fauna associated with environmental contamination of APIs, the releases of antimicrobial compounds from production facilities also raises concerns for promotion of antibiotic resistance development and dissemination, an event not confined or restricted to the area or even country of emergence but a potential threat to human health worldwide.

In a clinical setting, an organism is said to be resistant if the type and concentration of antibiotics previously used for treatment is no longer able to cure an infection. From a biological point of view, organisms which are less susceptible

to antimicrobials than the corresponding wildtype have developed resistance (*i.e.* the minimal inhibitory concentration, MIC, for the antibiotic has increased), irrespective of treatment failure or success. Over the past decades the rise in microbial resistance and the clinical consequences thereof have become increasingly evident. Today, the cost of antibiotic resistance is estimated to 1.5 billion euros a year and suggested to cause thousands of premature deaths, in Europe only (ECDC/EMEA 2009). However, the ability of microorganisms to develop mechanisms to withstand the effects from antibiotics is a historically well-known phenomenon which was addressed already in the Nobel price lecture by Sir Alexander Fleming in 1945 (http://www.nobelprize.org/nobel_prizes /medicine/laureates/1945/fleming-lecture.html). Furthermore, the time lapse from the launch of a new antimicrobial substance until a significant portion of the target organisms have developed resistance features are generally no longer than a few years (Schmieder and Edwards 2012) and the development of new antibiotics have stagnated (Walsh 2003). There are numerous ways that antibiotics exert their effects but commonly include the disruption of one or more of the central processes in the microbial cells, *e.g.* cell wall synthesis, DNA synthesis and cell replication, mRNA transcription and protein synthesis. Consequently, a plethora of resistance mechanisms also exist including alterations or inactivation of the antibiotic substance or modification of target structure(s), leading to a diminished or even absent inhibitory effect. Another common resistance mechanism is to decrease the internal concentration of antibiotics which is achieved through *e.g.* increased efflux or reduced cell membrane permeability. Microbial tolerance may also occur through bypass of the pathway inhibited by the antibiotic.

Some bacteria are inherently resistant to certain antibiotic substances, *e.g.* aminoglycosides are poorly taken up during anaerobic conditions why obligate anaerobes are unsusceptible to these antibiotics (Bryan et al. 1979). Also, new resistance traits can arise from the continuous random mutations of existing bacterial DNA, features which are subsequently inherited to daughter cells. In addition to the common passing on of genetic information to the next generation, bacteria are also able to exchange genetic material among each other, even between evolutionary distant species (Musovic et al. 2006). The phenomenon is

referred to as *horizontal* gene transfer (to distinguish it from the common *vertical* transfer from parental generation to offspring) and believed to contribute to the extensive and rapid dissemination of antibiotic resistance.

Horizontal gene transfer

Horizontal gene transfer (HGT) includes the acquiring and sharing of new and additional genetic material which can be carried out through several routes. Direct transmission through cell-to-cell contact is called conjugation, while transfer of DNA via viruses is referred to as transduction. A third possibility is transformation which involves uptake of naked, exogenous DNA from the surroundings. Any selective advantage provided by the newly acquired DNA, *e.g.* increase of the tolerance against an antimicrobial substance during antibiotic exposure, will increase the likelihood for maintenance and spread of the new trait.

Genetic material transferred through conjugation is often in the form of extra-chromosomal, circular and self-replicative DNA elements called plasmids. Larger plasmids (approximately >30 kilo base pairs) may also carry the genes needed to initiate and carry out the conjugative process while plasmids who lacks theses genetic mechanisms must instead rely on co-mobilization through the transfer machinery already established by the self-transmissible plasmids (Smillie et al. 2010). A single plasmid can contain multiple resistance genes and several copies of identical plasmids are often found in the same cell.

Other genetic elements also associated with antimicrobial resistance are integrons which are genetic platforms specialized for capturing and expressing genes in the form of discrete gene cassettes. The integrons frequently contain arrays of gene cassettes which can be excised and incorporated in new genetic contexts within a genome or between cells via mobile elements (Stokes and Hall 1989). When several resistance genes are located on the same genetic element, which is often the case for both plasmids and integrons, exposure to any of the corresponding antibiotics simultaneously co-select for the maintenance of the other resistance genes as well. Both plasmids and integrons may therefore be considered as key contributors in the dissemination of antibiotic genes and promoters of multi-drug resistance.

The environmental resistome

Many of the antimicrobial compounds used in the clinic today are derivatives of natural molecules produced by the microbes themselves (Bennett 2008). Given the extensive period of time that the earth has been inhabited by microorganisms, their relatively short generation time and genetic plasticity, which enables rapid adaptations to new environmental conditions, it seems logic that also strategies to evade the harmful effects from antibiotic exposure have evolved in nature.

In recent years, antibiotic resistance genes (ARGs) have been detected in pristine environments, including 30,000 year-old permafrost (D'Costa et al. 2011), sediments sampled far below land surface (Brown et al. 2008) and a cave isolated for millions of years (Bhullar et al. 2012). Additional findings of ARGs have been reported from remote locations with presumed minimal influence from anthropologic activities including antibiotic production and usage (Allen et al. 2009; Bartoloni et al. 2009). The extensive variability among environmental bacteria and the molecules they produce and excrete indicate that the substances used as antibiotics today have other and/or additional native functions *e.g.* as signal molecules for communication between cells and transcription modifiers at the lower concentrations often found in the environment, (Aminov 2009; Davies et al. 2006). The interpretation of antibiotics as versatile and multipurpose compounds is favored by the findings of ARGs in pristine environmental contexts. However, supposing the mechanisms for antibiotic resistance were evolved long before the industrial era of antimicrobial production, there is an increasing body of evidence indicating that the human use, overuse and misuse of antibiotics for the past decades is the major driver for the accelerating abundance and dissemination of ARGs during the past decades (Gaze et al. 2013; Knapp et al. 2009; Wellington et al. 2013). Nature would in this perspective serve as a giant reservoir of resistance genes and resistant bacteria (D'Costa et al. 2006). Highly similar or even identical ARGs, inhibiting antimicrobial substances with different modes of action, have been found in both environmental and pathogenic bacteria (Poirel et al. 2002; Poirel et al. 2005; Forsberg et al. 2012), emphasizing the potential shared resistome. Under a selection pressure from a combination of chemical compounds and pharmaceuticals or from antibiotics alone, *e.g.* caused by releases from drug production facilities, the environmental resistome could not

only be responsible for the maintenance and enrichment of ARGs but also function as a spawning ground for the development of new resistance genes. Resistance traits within the environmental resistome may thus have the potential to reach human pathogens and ultimately lead to clinical failure (Figure 2).

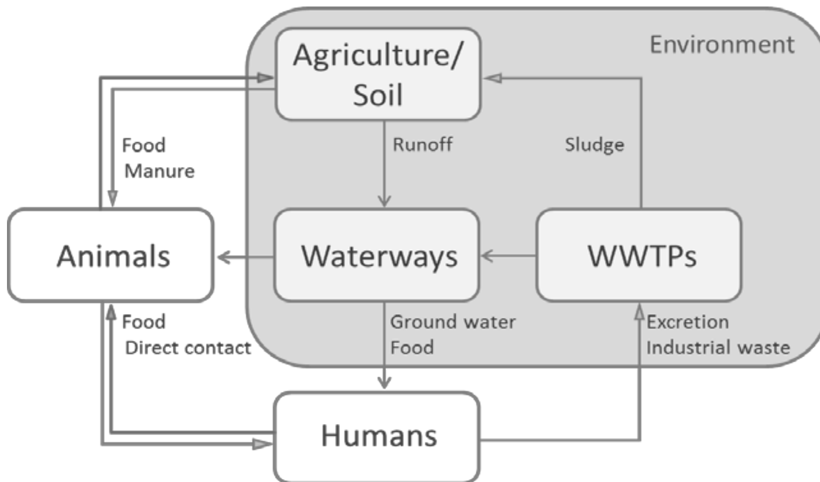

Figure 2. Schematic indicating the potential recycling of antibiotic resistant bacteria and resistance genes between humans, animals and the outer environment. In addition to maintenance of already circulating bacteria and genes, new resistance features may emerge and spread under a selective pressure, e.g. antibiotic treatment or environmental contamination. WWTP- waste water treatment plants. Inspiration from Schmieder et al. (2012).

Environments where bacterial density, antibiotic selection pressure and abundance of resistance genes are high, *e.g.* waste water treatment plants, are pointed out as hotspots for the recruitment, maintenance and spread of ARGs (Baquero et al. 2008; Rizzo et al. 2013). Additional studies in such milieus can aid in elucidating the potential effects on environmental bacteria exposed to high selection pressures from pharmaceutical pollution and guide future risk evaluations for human health.

Quinolone antibiotics and quinolone resistance

Of all the pharmaceuticals detected in the effluent from the waste water treatment plant in Patancheru, the fluoroquinolones (FQs) stood out as the most common class of drugs detected in high concentrations (Larsson et al. 2007; Fick et al.

2009). The first quinolone antibiotic was nalidixic acid introduced in 1962 and used for treatment of urinary tract infections but when the structural backbone was further developed including the addition of a fluorine atom, the treatment spectrum was significantly increased (Ruiz 2003). Today FQs are one of the most commonly prescribed drugs, including for veterinary purposes (Acar and Goldstein 1997), due to their broad-spectrum activity and relatively low cost after the introduction of generic brands.

The targets of FQs are the bacterial topoisomerase II enzymes DNA gyrase and topoisomerase IV which are both capable of inducing double-stranded DNA breaks, necessary for relieving DNA supercoils formed during replication, and for the appropriate separation of newly replicated daughter chromosomes (Drlica and Zhao 1997; Nordmann and Poirel 2005). Following passage of DNA strands, the topoisomerase enzymes are normally able to religate the broken DNA. However, FQs exert their bactericidal effects by stabilizing the topoisomerase II-cleaved DNA-complex (Drlica and Zhao 1997; Vetting et al. 2006).

Significant fluoroquinolone resistance has been reported from different parts of the world (Robicsek et al. 2006; EARSS 2008; Cheng et al. 2012) and low resistance rates are seen in countries with regulated and limited usage of fluoroquinolones in humans and food-producing animals (Cheng et al. 2012). High-level resistance to FQs commonly results from mutations in the so called quinolone-resistance-determining regions of target genes, *i.e. gyrA*/*gyrB* and *parC*/*parE*, encoding subunits of DNA gyrase and topoisomerase IV respectively (Jacoby 2005). Quinolone resistance can also occur through decreased intracellular drug accumulation via upregulation of efflux pumps and increased bacterial impermeability (Ruiz 2003; Poole 2005).

Furthermore, a plasmid-borne quinolone resistance mechanism was also discovered in 1998 (Martinez-Martinez et al. 1998). The protein, termed Qnr for **qu**i**n**olone **r**esistance, was shown to bind to and protect the topoisomerases from the inhibiting effects from quinolone drugs (Tran et al. 2005a; Tran et al. 2005b). Since then, numerous *qnr* genes have been detected and based on nucleotide or corresponding amino acid sequence identity and similarity (Jacoby 2008, qnr nomenclature), six *qnr* gene families; *qnrA,* (Martinez-Martinez et al. 1998), *qnrS*

(Hata et al. 2005), *qnrB* (Jacoby et al. 2006), *qnrC* (Wang et al. 2009) and *qnrD* (Cavaco et al. 2009), *qnrVC* (Pons et al. 2013) have been described. The *qnrB* gene family is hitherto the most diverse, containing over 70 variant alleles (Jacoby et al. 2008) (www.lahey.org/qnrStudies/). The plasmid-mediated *qnr* genes have likely been transferred to human pathogens from environmental bacteria and for the mobile *qnrA*, *qnrB* and *qnrS* genes the chromosomal origins in aquatic bacteria have been specified (Poirel et al. 2005; Jacoby et al. 2011; Cattoir et al. 2007).

The Qnr proteins belong to the pentapeptide repeat protein family which is distinguished by a five amino acid consensus sequence, represented by the 1-letter amino acid code as A(D/N)LXX, repeated throughout the protein (Bateman et al. 1998). The detailed molecular mechanism for the resistance phenotype seen in bacteria expressing Qnr proteins is not yet fully understood. It has been suggested that the Qnr proteins fold into structure resembling of DNA and thereby compete for the binding to topoisomerases, thus blocking quinolones from forming the lethal quinolone-topoisomerase-DNA complexes (Hegde et al. 2005; Robicsek et al. 2006). However, the theory has been questioned for failing to satisfactorily explain how the bacterial replication can proceed when the topoisomerases are blocked and another model propose that Qnr proteins interact with and destabilize the quinolone-DNA-topoisomerase complexes, releasing the antibiotic and freeing the topoisomerase enzyme to exert its central task of DNA rejoining (Vetting et al. 2011; Xiong et al. 2011). Also, to reach high level quinolone resistance often at least double mutations of target *gyr* and *par* genes are needed (Strahilevitz et al. 2009). The resistance phenotype conferred by *qnr* genes alone may not reach the clinical breakpoints for fluoroquinolones but still be of vital importance because the decreased quinolone susceptibility can facilitate the emergence of higher-level resistance (Martinez-Martinez et al. 1998; Jacoby 2005; Nordmann and Poirel 2005; Robicsek et al. 2006).

The discovery of *qnr* genes was somewhat unexpected since the quinolone drugs are completely synthetic drugs. However, natural quinolone compounds have been found, some of them having pharmaceutically relevant characteristics (Heeb et al. 2011). Other studies have shown that natural quinolones can be involved in quorum signaling (Dubern and Diggle 2008) and that subinhibitory

concentrations of the synthetic FQ norfloxacin can act as a signaling agent in bacteria (Linares et al. 2006). Also, natural quinolones can induce *qnr* mRNA expression (Kwak et al. 2013) which may imply a natural protective function of these proteins against DNA damaging agents.

AIMS

The overall aim of this thesis was to increase our knowledge on environmental pollution from drug manufacturing. The included studies have focused on investigating both local/acute effects from pharmaceutical contamination and exploring the potential global/long-term consequences from antibiotic resistance development and dissemination. The ambition is to provide a broad perspective on possible risks.

Specific aims

Paper 1 & 2	Investigate whether short-term exposure to a treated effluent from bulk drug production, containing a mixture of active pharmaceutical ingredients, is toxic to aquatic and terrestrial vertebrates. Explore the mechanisms of action and suggest potential biomarkers for short-term exposure to effluent from drug manufacturing.
Paper 3	Explore the genetic basis for antibiotic resistance in Indian river sediment exposed to exceptionally high levels of antibiotics.
Paper 4	Determine the concentrations of antibiotics and study potential effects on antibiotic resistance genes, in well water and irrigated soil contaminated by wastewater from bulk drug production.
Paper 5	Investigate the potential link between local environmental fluoroquinolone pollution and the prevalence of quinolone resistance genes (*qnr*) in human gut microflora.

A comprehensive account of all aspects of pharmaceutical contamination of the environment as well as antibiotic resistance development is beyond the scope of the present thesis.

METHODOLOGICAL CONSIDERATIONS

Environmental sampling

The effluent used for the experiments with rainbow trout and rat (Paper 1 and Paper 2, respectively) was sampled on two sequential days in November 2006 from PETL (Patancheru Enviro Tech Ltd.), a plant treating waste water from numerous bulk manufacturers in Patancheru, India. The effluent was kept in light-protected bottles in -20°C until the start of the experiments and was reanalyzed before the rat exposure study to ensure that pharmaceutical concentrations were comparable to those measured at the sampling occasion.

The Indian river sediments analyzed in paper 3 and paper 5 was sampled on the 28th of March 2008 at six locations, two sites upstream from PETL, one close to the discharge site and three sites downstream from the plant, the furthest approximately 17 km away from PETL (Figure 3).

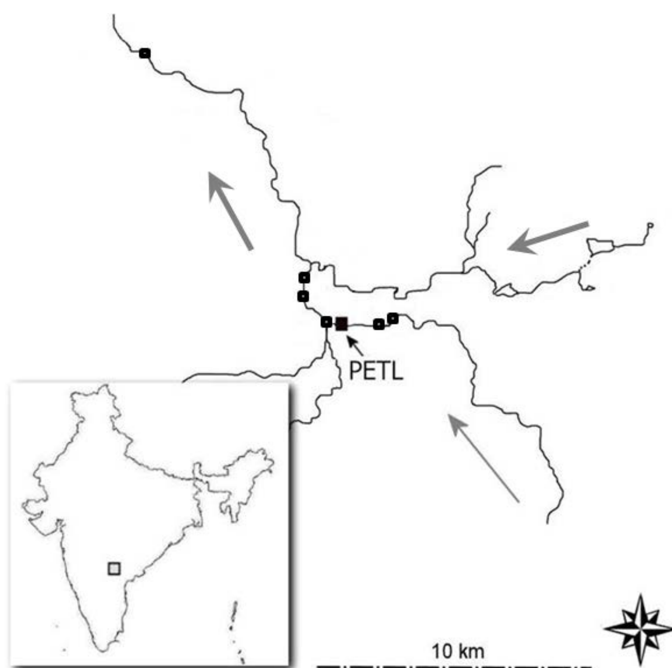

Figure 3. Sampling points for Indian sediment analyzed in paper 3 and paper 5. For exact sampling coordinates, see paper 3. Picture modified from Fick et al. (2009).

From each site, six sub-replicates were sampled within a meter apart. The Swedish reference sediment samples used for analysis in the same papers were collected from a municipal sewage treatment plant in Sweden on 3rd May 2009, at five sites, 5-100 meters upstream from the discharge point and six sites, 25-230 meters downstream from the discharge site, respectively.

The soil and well water samples analyzed in paper 4 was collected in 15 villages in the vicinity (up to 36 km) of PETL at two time points, 12th January and 13th June 2011, respectively (Paper 4; Figure 1). At the January sampling, four of the bottles with well water were lost during transportation to Sweden why n=11 for this sample type and occasion. It should be noted that the monsoon season had begun about a week prior to the June sampling with likely affected both soil sites and water levels in wells. Screening and concentration analysis of chemical substances, including pharmaceuticals, present in sediment, well water and soil aimed to establish a link between the degree of environmental pollution and the prevalence and abundance of specific genes or larger genetic features, *i.e.* plasmids, integrons.

Animal models

Two animal models were used in the studies included in this thesis to investigate direct effects for local fauna from effluent exposure. The model species represent one aquatic vertebrate, as water-living organisms are often exposed to pharmaceutical residues and APIs ending up in waterways, and one terrestrial vertebrate, facilitating the extrapolation of exposure responses for estimating the potential risks for land-living mammals including humans.

Fish populate diverse environmental niches throughout the world and commonly represent a valuable resource. As the knowledge about their physiology and exposure responses is growing, fish are often used as sentinel species in biomonitoring programs where environmental status is investigated through surveillance of biota. In one of the exposure study included in this thesis (Paper 1), rainbow trout (*Oncorhynchus mykiss*) was chosen the model organism. The same species have been used in preceding studies in our lab providing experience regarding exposure experiments to single substances (Stephensen et al. 2002; Sturve et al. 2005), as well as complex STP effluent (Sturve et al. 2008)

confirming that *O. mykiss* is thriving under laboratory conditions. Also, a microarray had been developed and optimized for the fish and the in-house Geniom microarray platform (Gunnarsson 2009).

In attempts to reduce biological variation not due to the different treatments (effluent exposure or not), juvenile fish was purchased from a local vendor where age and health status prior to the experiment are generally more easily controlled than when wild fish are used. At the fish farm, and during the acclimatization period (5 days), the fish were fed commercial trout pellets. However, in a laboratory setting a strong hierarchy is commonly established in aquaria, likely leading to unequal amounts of food ingested among individual fish. To avoid that the potential responses to effluent exposure were affected by socially-induced variations in food intake the fish were not fed during the 5-day experiment, a starving time period which *O. mykiss* is known to cope well with (Kullgren et al. 2010).

Effluent was thawed the day before the start of the exposure experiment and kept in light-protected containers and a flow-through water system was used to better resemble natural conditions with a continuous addition of effluent. In fish exposure experiments, there are limitations regarding fish density in aquaria, why the size and number of fish have to be compromised. As the volume of effluent to be added to exposure aquaria was rather limited, a short exposure time and a relatively high dilution of effluent, 1:500, was used. A previous study has shown that the growth of tadpoles was significantly inhibited at the same effluent concentration (Carlsson et al. 2009).

There is a broad knowledge of the basic physiology of rat *(Rattus norvegicus)*, the organism selected as the terrestrial model species in paper 2. Also, rats have offered a lot of information in studies associated with drug development why there is much data on responses and effects after pharmaceutical exposures. In this study male rats of the common laboratory strain Sprague-Dawley were used, aged five to six weeks. During the whole experiment, the animals had free access to tap water and regular chow. To better simulate the prerequisites at a worst case scenario for animals living in the environmentally contaminated area in Patancheru, an experimental set-up where effluent was the only available water

source would have been preferred. However, due to the foul smell of the water the rats would potentially not drink at all or enough for maintaining good health which would have hampered the initial aim of discovering early signs of exposure and adverse effects from the exposure. Instead, each rat was tube-fed an equal volume of either effluent, single substance solution or tap water, once daily by experienced animal technicians.

At the termination of both fish and rat exposure studies, blood was drawn and various tissues harvested. In the second part of the rat exposure experiment, the concentration of 97 pharmaceuticals was measured in blood serum sampled 1h and 24h after the fifth and final tube-feeding respectively. The drug levels were measured to investigate whether, which and to what extent pharmaceuticals were absorbed via the intestine after oral administration and to correlate potential alterations in mRNA abundances and physiological responses to the absorbed dose of pharmaceuticals. Also, the chemical analysis could suggest if the drugs' pharmacokinetics were synergistically or antagonistically affected when administered in a complex effluent compared to in single substance solutions.

The weight of the collected organs in both fish and rat was noted and somatic indices were calculated but no treatment effects could be seen on neither of the tissues, nor on whole body weight and length. No animals showed obvious signs of discomfort during the exposure experiments and there was no need for pre-termination of the studies.

For both the fish and rat studies, standard protocols for metabolite concentrations and enzyme activities in blood serum/plasma were used to indicate (adverse) effects from pharmaceuticals and disturbances of specific organ functions. The global analysis of mRNA abundance focused on the liver due to its function as the central organ for detoxification and transformation of exogenous substances, xenobiotics. The RNA was quality checked prior to microarray and qPCR assays ensuring minimal degradation and contamination of *e.g.* solvents during extraction, which otherwise can disturb and introduce noise in downstream applications and analyses. Experiments were approved of by the local animal ethics committee in Gothenburg (application numbers 36-2007, 5-2008 and 155-2011, respectively).

Human sampling

Indian stool was collected from 11 villages in the Patancheru area. Villages denoted I-VIII were sampled on the 16th and 18th of July, 2010 (n=86), with help from the local non-governmental organization Gamana. Two of the villages (Gandigudem and Rai Bollaram Thanda) were re-sampled on the 18th of April 2011, although only a single individual was sampled twice. Also, at this time point, samples from three additional villages were collected, making a total of n=71. Of all 157 participants, 53% were women/girls and the age span ranged from 4-75 years old.

Swedish stool samples were collected between April 2010 and May 2012 (n=37) and 78% of the participants were women. Compared to the Indian study group, the Swedish participants were more homogenous in age, 22 to 34 years old, because samples were collected from a group of medicine students. All sampling events are summarized in figure 4.

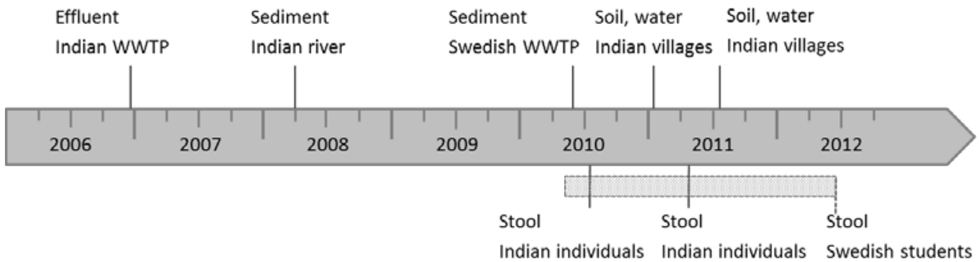

Figure 4. Time line of sampling events for the different matrices analyzed in the present thesis. Stool samples from Swedish students were collected during two years (dashed line) while other samplings took place during a single or consecutive days. WWTP-waste water treatment plant.

Stool samples from individuals stating they had received antibiotic treatment during the last six months prior to sampling were excluded from the study. All participants gave informed and voluntary consent to sample collection. For Indian stool sampling, institutional ethical clearance was obtained by Dr Y Shouche, Pune University. Stool sampling in Sweden were approved by the regional ethical review board in Umeå (2011-357-32M).

Chemical measurements

The concentrations of pharmaceuticals were determined using gas or liquid chromatography (GC or LC) coupled to tandem mass spectrometry, (MS/MS), in effluent (Paper 1), rat serum (Paper 2), river sediment (Paper 3) and soil and well water (Paper 4).

GC/LC-MS/MS is, as the name indicates, several analysis techniques in series, and can be used for various chemical applications where particles or molecules in a composite sample are separated for individual identification. In short, the sample of interest is carried by means of a mobile phase which can be a liquid or a gas through a solid medium and, depending on their particular chemical properties, the analytes in the mixture will move by different speeds, and thus be separated. The molecules are then electrically charged, led through an electromagnetic field and sorted according to their specific mass to charge ratio (m/z). The ions are detected and their identity and relative abundances can be determined by comparing the pattern and height of peaks in a resulting chromatogram to the results from previous runs with known reference molecules of specified concentrations. The sensitivity of LC is not as pronounced as with GC but can be increased by using several mass spectrophotometers in tandem as in several of the studies included in the present study. To minimize the risk for carry-over between sample runs blanks were run in between and spiked reference samples were used for obtaining recovery data.

Analysis of genomic DNA and mRNA expression

In the studies included in this thesis, both analysis of messenger RNA (mRNA), through microarray and qPCR analysis (Paper 1 and Paper 2) as a measure of gene activities and responses to effluent exposure, and DNA, through qPCR (Paper 4 and Paper 5), determining the prevalence and copy number of specific genes, have been performed.

Microarrays

The explorative nature of microarrays has opened up new possibilities within the biology research field. The ability to analyze the abundance of thousands of

mRNAs simultaneously enables a more unprejudiced hypothesis generation. The extensive studies during drug discovery and development have accumulated a lot of knowledge regarding pharmaceutical interactions, but there still are potential risks for adverse and unexpected effects on wildlife associated with unintentional drug exposure as *e.g.* additional or other targets may be affected (Fent et al. 2006), making microarrays a valuable tool for ecotoxicological studies.

In the studies included in this thesis, two different microarray platforms have been used; the Geniom/RT-analyzer platform from febit (Paper 1) and the GeneChip Rat Genome 230 2.0 Array from Affymetrix (Paper 2). Because ready-made arrays for species relevant for environmental research, including rainbow trout, is lacking, a custom microarray was designed. The Geniom array used for analysis of the rainbow trout transcriptome has previously been developed and optimized in-house and evaluated to give results comparable to several commercially available arrays (Gunnarsson 2009). In contrast, for the analysis of global hepatic gene transcription in rats, for which the complete genome has been sequenced, a well-established array available on the market was chosen and the experiments were run at the Swegene Centre for Integrative Biology at Lund University.

Despite slight variations in the experimental protocols, the procedures for the microarray analyses in the present studies are quite similar. In short; mRNA is extracted from the liver and converted into amplified and labeled RNA. Upon hybridization to complementary probes attached on the array chip surface, a fluorescent signal is detected. The light intensity depends on the amount of mRNA in the sample and hence gives a representation of the degree of gene transcription.

The large amount of data generated from a microarray experiment needs to be normalized to reduce the impact from technical artifacts *e.g.* unequal hybridization efficiency, and enable comparisons between samples. Furthermore, many of the numerous transcripts analyzed in parallel on an array chip are not independently regulated and the number of biological replicates included in a study is generally low due to the relatively high cost of microarray experiments. To avoid an inadequate estimation of variance among data points, modified statistical

tests intended for microarray analysis are used to identify differentially expressed genes. Also, due to the high number of tests performed in the microarray analysis, the false discovery rate was calculated to estimate the proportion of false positives, *i.e.* genes that appear significantly regulated in a statistical test due to chance and not because of a treatment-induced effect (Benjamini and Hochberg 1995).

The output of microarray analysis, a list of genes whose expression is altered between differently treated groups, is generally too complex for immediate interpretations. Numerous bioinformatics tools are available to aid in identifying patterns in the data to make it biologically meaningful, including Gene Ontology (GO) (Ashburner et al. 2000) and the Kyoto Encyklopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2008) which were used in paper 1. These resources classify genes and gene products depending on their cellular location, molecular function and association with specific biological processes and pathways and were used for identifying overrepresentation of particular GO terms and/or KEGG pathways among regulated genes. If annotations for the species of interest are lacking, as in the case with rainbow trout, information from genes and gene products with high sequence similarity in other species may be used. However, because the number of available annotations differs between organisms and the conclusions drawn are exceedingly dependent on the information already accessible in the database why the reference species should be chosen with care.

Quantitative polymerase chain reaction

Hypotheses generated from microarray analysis are generally validated through additional assays. Quantitative polymerase chain reaction (qPCR) is a powerful tool because of its sensitivity enabling identification and quantification of also low-copy number genes or mRNA templates provided that the assay is optimized and the primers well-designed. In the studies included in this thesis, primers for target genes were either adopted from the literature or custom-designed using available software. Based on the length and nucleotide sequences of the proposed primers the programs estimate the risks for self-ligation enabling selection of primers less prone for dimerization and formation of secondary structures. The templates for primer design come from previously published sequence data. As the genome of rainbow trout is not fully sequenced, data from other evolutionary

closely related species was occasionally used for identifying conserved regions suitable for primer design. For the rat study, preformulated and evaluated qPCR primers and probes were purchased.

The previously published primers for *sul2*, *intI1* and *16S rDNA* used in the bacterial experiments targeted genes in environmental samples and the *qnr* primers were designed using the templates for the first member of each respective *qnr* family (available at http://www.lahey.org/qnrStudies/). Ideally, each *qnr* primer pair would target all known alleles within the corresponding family but after trying out degenerate primers (Guillard et al. 2011), the custom-designed primers performed better at discriminating between sample concentrations and were therefore used for subsequent analysis. When all *qnr* sequences included in the database at http://www.lahey.org/qnrStudies/ were aligned, the chosen *qnr* primers did not match perfectly to all variants reported within each gene family. However, it is possible that the primers are able to recognize at least some of the alleles depending on the number and location of the mismatches between primers and genes. Because of the large amounts of DNA required for the metagenome sequencing (Paper 3), the starting material had to be amplified before analysis. Due to the known drawback of biased amplification of DNA in Repli-G and other whole genome amplification approaches (Abulencia et al. 2006; Pinard et al. 2006), qPCR analysis was performed on non-replicated material why the amount of DNA used in each reaction was relatively low.

In paper 1 and paper 2, the relative difference in mRNA abundances between treated and control animals were analyzed. To correct for potential dilution and pipetting errors having caused unequal loading of starting material, results were normalized against the levels of house-keeping genes. These genes (*ubiquitin* and *tubulin alpha,* and *b-actin* and *b2-microglobulin* for the fish and rat exposure studies respectively) were obtained from the literature or identified during the microarray analysis for being expressed at levels similar to target genes, having low variability between individual animals and not being affected by the treatment.

In paper 4 and paper 5, DNA copy numbers were determined using standard curves; serial dilutions with known concentrations of the target gene, which were included in all runs. Additionally, the gene for 16S rRNA, which contains regions

highly conserved between different bacterial species, was analyzed for all samples and used as the reference gene to which the copy numbers of the target genes was related.

For all qPCR analyses, the specificity of the amplified product was analyzed by the generation of a dissociation curve. Also, in the gene expression experiments, samples where no reverse transcriptase enzyme had been added were included in all runs to check for genomic contamination.

Metagenome sequencing

Studying ARGs from the environment have traditionally involved culturing the bacteria under the selection pressure from an antimicrobial agent. However, the great majority of environmental bacteria are not easily grown with the current laboratory methods (Hugenholtz et al. 1998; Streit and Schmitz 2004). A more adequate representation of the variety of genes present in the environmental resistome can result from additional methods circumventing the cultivating step. In metagenome sequencing, (in theory) all DNA in a sample is extracted, fragmented and sequenced in a high-throughput manner (Wooley et al. 2010). The vast amount of short-read data generated during the sequencing is, by means of a powerful computer and a skilled bioinformatician, assembled into longer DNA stretches and compared to previously reported sequences. The need for reference data makes the metagenome sequencing approach suboptimal for discovering novel ARGs; however the technique has successfully been used for identifying genes with high sequence similarities and thus potentially similar functions to already known ARGs (Boulund et al. 2012). So far, metagenome sequencing is mainly used for identification and quantification of multiple ARGs in complex samples (Forslund et al. 2013; Shi et al. 2013; Zhang et al. 2011). Even though the technique is not as sensitive as *e.g.* PCR, the wide range of genes analyzed in parallel may provide additional information regarding *e.g.* taxonomic and functional conditions of the microbial communities present in the sample.

Enzymatic assays

Effects on gene expression, measured as mRNA abundances, do not necessarily lead to an altered protein production, even though studies have estimated the

levels to be fairly congruent (de Hoog and Mann 2004; Lu et al. 2007). As most functions in a cell are dependent on proteins, any treatment effects seen on this higher organization level is more likely to be biologically significant.

The results from the microarray and subsequent qPCR assays in paper 1 indicated treatment effects on *e.g. cyp1a* and oxidative stress-responsive genes and the enzyme activities of a set of corresponding proteins was further analyzed. The activities of Cyp1a, glutathione-S transferase, glutathione reductase and catalase were determined by adding the appropriate reagents to homogenized liver fractions and spectrophotometrically observe the degradation of substrate or the generation of product in a time series (Carney Almroth 2008).

RESULTS AND DISCUSSION

Pharmaceutical contamination in Patancheru

The findings of unprecedentedly high concentrations of several APIs in the effluent from PETL (Larsson et al. 2007; Fick et al. 2009) demonstrate that the treatment of process water, common to a large number of drug manufacturers in the Patancheru area, is unsatisfactory. It also indicates that the effluent from PETL is a source for the pharmaceutical pollution observed downstream from the waste water treatment plant (Fick et al. 2009; Paper 3). Moreover, the detection of considerable levels of APIs in river water upstream from PETL and in nearby lakes (Fick et al. 2009) indicates additional contamination sources. Indeed, the Andhra Pradesh Pollution Control Board reports of several unauthorized events of dumping of industrial waste in the Patancheru area (Boralkar et al. 2004; www.toxicslink.org/docs/SCMC_Visit_AP.doc). Up to 0.9 mg/g organic matter of ciprofloxacin was detected in river sediment sampled downstream from PETL (Paper 3). Moderate levels (up to 7.1 µg/g organic matter) was found in upstream river sediments while no FQs could be detected in sediments sampled up- and downstream from a Swedish waste water treatment plant. This is in agreement with surface water levels up and downstream from PETL, determined from grab samples (Fick et al. 2009). The pharmaceutical contamination has also reached the groundwater in the area (Fick et al. 2009; Paper 4). In samples from 2008, several drugs were detected in high concentrations (>1 µg/L) in well water in nearby villages, ciprofloxacin being found in all wells in concentrations up to 14 µg/L (Fick et al. 2009). The Andhra Pradesh Pollution Control Board reported that since July 2009 a gradually increasing proportion of effluent have been transported through an 18 km pipe line from PETL to another waste water treatment plant, reaching 100% of the discharged waste water in March 2010 (APPCB 2010). In January and June 2011, well water from 15 villages, including the six villages previously studied, was re-sampled and the concentration of FQs was analyzed with LC-MS/MS. FQs were detected in all well water samples from villages located <3km from previously documented contaminated waterways (Paper 4) with up to 770 ng/L and 180 ng/L of ciprofloxacin in samples collected in January and June respectively. Even though these levels are higher than the

concentrations generally found in treated sewage effluents (Lindberg et al. 2005), in comparison to the levels detected in 2008, the concentration of FQs in well water had decreased (Table 2).

Table 2. Summary of detected concentrations of ciprofloxacin in environmental matrices in the Patancheru area. Concentrations of water samples given in ng/L and sediment/soil samples in ng/g dry weight. PETL-Patancheru Enviro. Tech Ltd. WWTP-waste water treatment plant. ND-not detected.

Sample type	Sampling date	[Ciprofloxacin] (μg/L)[a] or (ng/g d.w)[b]	Reference
WWTP effluent			
PETL effluent	November, 2006	28,000-31,000[a]	Larsson *et al.* 2007
PETL effluent	March, 2008	14,000[a]	Fick *et al.* 2009
Surface water			
Lake in the Patancheru area	March, 2008	2,500-6,500[a]	Fick *et al.* 2009
River upstream of PETL		12[a]	
River downstream of PETL		10-2,500[a]	
Sediment/soil			
River sediment upstream of PETL	March, 2008	400-1,000[b]	Kristiansson *et al.* 2011 (Paper 3)
River sediment downstream of PETL		1,700-54,000[b]	
River sediment up- and downstream of a Swedish WWWTP	May, 2009	ND	
Soil in villages in the PETL area	January, 2011	3-17[b]	Rutgersson *et al.* manuscript (Paper 4)
	June, 2011	1,400-1,900[b]	
Well water			
Villages in the Patancheru area	March, 2008	0.04-14[a]	Fick *et al.* 2009
Villages in the Patancheru area	January, 2011	0.02-0.7[a]	Rutgersson *et al.* manuscript (Paper 4)
	June, 2011	0.04-0.3[a]	

For the villages located near PETL (Baithole, Pocharam and Ganapahigudem), this apparent decrease in concentrations over time could be a potential consequence of the stated decreased emissions from the treatment plant due to the rerouting of waste water to another river system. However, the FQ concentrations in villages located upstream from PETL is likely more affected by the degree of dumping and emissions from isolated production facilities into nearby waterways. In most of the villages in Patancheru, well water is no longer used as a drinking

water source (Fick et al. 2009) but is still used for cleaning clothes, bathing etc. The well water is also still used for irrigation of arable land (primarily rice fields). It was therefore of importance to investigate whether farmland soil contained antibiotics as contaminated crops can be a possible route for unintended exposure of antibiotics to terrestrial vertebrates including humans. Albeit at generally low levels, FQs were detected in all, and only in those, soil samples from villages where FQs were also detected in well water (Paper 4; Suppl. Table 1). This suggests that irrigation with contaminated ground water have polluted the local soil with antibiotics. The levels of ciprofloxacin detected in January soil samples (up to 14 ng/g dry weight) is in the same range as previously published data on concentrations found in soils irrigated with reclaimed water (Li et al. 2011; Shi et al. 2012). For soil collected in June, FQ were detected in two samples only (ciprofloxacin in Baithole and Sultanpur). Nevertheless, the concentration was 1.4 and 1.9 µg/g dry weight which is similar to the levels found in sludge from several waste water treatment plants (Golet et al. 2002). It cannot be excluded that the FQ levels in soil were higher at an earlier time point when the antibiotic well water concentrations were further elevated (Fick et al. 2009). However, the complexity of the soil matrix makes it difficult to estimate the degree of bioavailability of the detected FQs. It should also be noted that we have focused on the levels of FQs as a proxy for general contamination and it is therefore possible that the concentration patterns of other compounds are different from the trends of FQ levels in well water and soil in Patancheru.

Direct effects

The direct effects of PETL effluent exposure on biota was in this thesis assessed in vertebrates using two species; rainbow trout (*Oncorhynchus mykiss*) (Paper 1) and rat (*Rattus norvegicus*) (Paper 2) serving as models for aquatic and terrestrial vertebrates, respectively.

After five days exposure of highly diluted effluent (1:500) in a flow-through system, the concentration of phosphate and cholesterol was significantly increased ($p=0.008$ and 0.02 respectively) in blood plasma of exposed fish. Elevated phosphate levels can be an indication of renal damage in mammals (Berner and Shike 1988), and hyperphosphatemia in fish have been observed after exposure to

e.g. chlorinated hydrocarbons (Gill et al. 1991; Singh et al. 1996). The increased concentration of blood plasma cholesterol could be associated with some gene regulation patterns as measured by the microarray. For example were the GO-terms triacylglycerol metabolic process (GO: 0006641) and cholesterol catabolic process (GO:0006707) overrepresented on the top 300 and 600 list of most significantly regulated genes respectively. However, how or if increased plasma cholesterol affects the well-being of fish is unknown. Additionally, the global hepatic mRNA expression analysis demonstrated an upregulation of *cyp1a*, a member of the cytochrome P450 (cyp) family. The encoded Cyp enzyme is involved in the break-down of endogenous as well as xenobiotics substances, including pharmaceuticals and the induction of *cyp1a* mRNA and enzyme activity is used as biomarkers of exposure to pollutants (Goksøyr and Förlin 1992; van der Oost et al. 2003; Valavanidis et al. 2006) A wide range of chemicals are able to induce *cyp1a* mRNA expression including polyaromatic hydrocarbons, dioxin-like compounds such as polychlorinated biphenyls (Whyte et al. 2000) and pharmaceuticals (Lee et al. 2006). The substrates for Cyp1a metabolism can often induce *cyp1a* mRNA via the aryl hydrocarbon receptor (AhR) even though more mechanisms for upregulation have been identified (Hu et al. 2007). A prolonged activation of AhR can have toxic effects on a range of organs (Gonzalez Ward 1996) but does not always lead to dioxin-like toxicity (Hu et al. 2007). In the present exposure study, in addition to *cyp1a*, some other genes also responsive to AhR activation were altered in exposed fish while others were not affected. Thus, it is unclear whether the observed effects after effluent exposure are mediated through AhR induction. The effluent from PETL has not only been screened for pharmaceuticals, (Larsson et al. 2007; Carlsson et al. 2009; Fick et al. 2009) but also for a range of other chemicals, including solvents and pesticides (Carlsson et al. 2009). No compound was detected in a concentration likely to be solely responsible for the induction in Cyp1a activity observed in the fish exposure study. Due to the extensive number of APIs and chemicals that exists that were either not analyzed or for which effect data after exposure in fish is lacking, it is possible that a substance not included in the measurements was in fact present in the effluent causing the induction of Cyp1a. A mixture effect from multiple compounds, individually below detection limit, is also possible. Fluoroquinolones, which were detected in high concentrations in the PETL

effluent, have been shown to inhibit hepatic Cyp1a activity in human and rats (McLellan et al. 1996; Regmi et al. 2005). Exposure to about 2% effluent (*i.e.* 10 times higher than used in paper 1) has been shown to reduce Cyp1a activity (Gunnarsson 2009; Beijer et al. 2013). Due to the high dilution of effluent used in the fish exposure study (0.2%), the inhibitory properties of FQ are assumed low but it is possible that the detoxification process was hindered (by FQs or other compounds in the effluent) why Cyp1a substrates, which are often also *cyp1a* inducers, may have accumulated and induced gene transcription.

The upregulation of *cyp1a* mRNA as measured by microarray was confirmed by qPCR and Cyp1A induction was reflected by increased enzyme activity. The biotransformation activities from *i.e.* Cyp1 enzyme can lead to an increased production of reactive oxygen species, ROS, which in turn can induce oxidative stress and potential cell damage (Carney Almroth 2008). Indeed, the microarray and following qPCR analyses indicated effects on several oxidative stress-related genes why the hepatic levels of glutathione and the enzyme activities of glutathione-S transferase, glutathione reductase and catalase were analyzed. In contrast to the observed increase in Cyp1a activity, none of the other measured enzyme activities were induced in exposed fish nor was the concentration or the ratio of oxidized versus reduced glutathione altered between treatment groups.

The results from the microarray analysis also showed that *estrogen receptor 1* (*esr1)* was up-regulated in exposed fish, which suggests that the effluent contains substances with estrogenic effects. Raw human sewage is continuously added to the Indian treatment plant to maintain the efficacy of biological treatment (Larsson et al. 2007) and the concentrations of the naturally occurring estrogens estrone, estradiol and estriol in addition to the synthetic 17α-ethinylestradiol, commonly used in oral contraceptive pills, were analyzed in the undiluted effluent (Paper 1). Among these, only estriol was detected but, due to the high effluent dilution used in the exposure study, not in a concentration high enough to explain the *esr1* mRNA induction (Thorpe et al. 2003). In addition to *esr1*, the qPCR assay also measured the mRNA abundance of *vitellogenin* (*vtg*) a commonly used as biomarkers for estrogenic exposure in male and juvenile fish (Sumpter and Jobling 1995) and *zona pellucida protein 3* (*zp3*), both genes inducible by estrogens (Thomas-Jones 2003). The qPCR showed a significant induction in

mRNAs for *esr1* and *zp3* but not *vtg* which is in line with previous data reporting that hepatic gene induction of *zp3* is more sensitive to estrogen exposure than *vtg* (Celius et al. 2000; Thomas-Jones et al. 2003; Gunnarsson et al. 2007).

Many human drug targets are evolutionary conserved in other organisms including aquatic vertebrates (Gunnarsson et al. 2008), why unintended effects on wildlife are conceivable due to the high levels of numerous pharmaceutical substances in the PETL effluent. However, fluoroquinolone antibiotics, the group of drugs dominating the effluent, target binding sites on prokaryotic enzymes, and seem to confer low toxicity to aquatic vertebrates as previous studies observed no effects on zebrafish and frog embryos after exposure to 100 mg/L of ciprofloxacin for 3-4 days respectively (Halling-Sørensen et al. 2000; Richards and Cole 2006). However, studies on terrestrial vertebrates have shown direct adverse effects on skeletal formation in utero after ciprofloxacin exposure (Gerenutti et al. 2006; Stahlmann 2003) albeit at daily mg/kg administered doses. These effects are not found in human developing fetuses (Bar-Oz et al. 2009).

Rather subtle but clearly detectable effects were found in rainbow trout after short-term exposure to 0.2% of PETL effluent. With the approach taken, we were however not able to identify specific toxicants responsible for the observed effects. It is possible that unmeasured and potent substance(s) in the effluent is the causative agent(s) of the observed effects but it may also be a combination of numerous substances individually found in low levels or below detection limits. The results in paper 1 conform to data from (Carlsson et al. 2009) who demonstrated inhibited growth in developing tadpoles exposed to an equal dilution of the same effluent. Beijer et al (2013) exposed stickleback (*Gasterosteus aculeatus)* to 0.8% effluent and reported on induction of *cyp1a* mRNA in liver, gill and brain. Less than 7% of effluent was enough to reach EC_{50} thresholds in standard toxicity tests on plants (*Lactuca sativa*), water flea (*Daphnia magna*) and bacteria (*Aliivibrio fischeri*) (Larsson et al. 2007). Table 3 summarizes the observed effects from PETL effluent exposure experiments. Together, this data indicates that an extensive area in the Patancheru region is contaminated to the point where it affects aquatic wild-life. Additional studies may aid elucidating if the rerouting of PETL effluent will have any effects on the local flora and fauna in the Patancheru region.

Table 3. Summary of observed effects from PETL effluent exposure experiments.

Species	Tested dilutions	Effect parameter	Studied endpoints	Observed effects	Exposure time	Reference
Bacteria (*Aliivibrio fischeri*)	1.25-10%	3% (EC_{50})	Luminescence		15min	Larsson *et al.* 2007
Plant (*Lactuca sativa*)	1-50%	1.6-35% (EC_{50})	Emerging seedlings/ developed cotyledons		5 days	
Water flea (*Daphnia magna*)	0.6-10%	6.7-7.2% (EC_{50})	Immobility		48h	
Fish embryo (*Danio rerio*)	1-16%	2.7-8.1% (LC_{50})	Lethality		6 days	Carlsson *et al.* 2009
		3.6-7.8% (EC_{50})	Sublethal effects, *e.g.* developmental defects and presence of edema			
Tadpole (*Xenopus tropicalis*)	0.2-2%	0.2% (LOEC)	i. growth ii. developmental stage	i. Inhibited growth ii. reduced development stage at 2% effluent	14 days	
Fish (*Oncorhynchus mykiss*)	0.2%	0.2% (LOEC)	i. global hepatic mRNA expression ii. hepatic Cyp1a activity iii. metabolite concentrations and enzyme activities in plasma	i. effects on genes associated with xenobiotic metabolism (*e.g. cyp1a*) and stress response (*e.g.* oxidative stress and unfolded protein binding) ii. induced Cyp1a activity iii. increased phosphate and cholesterol levels	5 days	Gunnarsson *et al.* 2009
Fish (*Gasterosteus aculeatus*)	0.8-3.2%	0.8% (LOEC)	i. mRNA expression of *e.g. Cyp1* in gill, liver and brain ii. gill Cyp1a activity iii. time dependence of observed effects	i. induced *cyp1a* mRNA expression in all tissues ii. inhibited Cyp1a activity iii. Observed effects disappeared after 9 days in clean water	24h	Beijer *et al.* 2013
	2.5-40%	5% (LOEC)	Inhibition of Cyp1a activity gills		2h	
Rat (*Rattus norvegicus*)	1.5mL /day	LOEC>1.5mL /day	i. global hepatic mRNA expression ii. metabolite concentrations and enzyme activities in blood plasma	No overt effects	5 days	Rutgersson *et al.* 2013

In paper 2, rats (n=10+10) were orally exposed for undiluted effluent for five days through tube-feeding. Large-scale and explorative methods such as screening of blood metabolites and enzyme activities and microarray analysis of hepatic mRNAs were analyzed aiming to identify early and sub-lethal effects which could offer clues of the mechanism of toxicity previously described in aquatic vertebrates. Clear responses from the global hepatic gene expression analyses would have allowed comparisons to a microarray result database where mRNA patterns from rats exposed to hundreds of substances are collected (Ganter et al. 2005). The limited volume of effluent administered to the rats implied that the animals were exposed to less than 7% of the human defined daily dose (DDD; adjusted for body weight) for any of the individual APIs measured in the effluent. However, due to the potential presence of unidentified substances and possible combinatory effects, exposure effects were conceivable. No effects on clinical blood chemistry analyses were however observed in exposed rats. Furthermore, the microarray results showed few differences in mRNA levels between treatment groups, the alterations were generally small and indicated to contain a high degree of false positives (all genes had an estimated false discovery rate >88%).

In a parallel study, rats were tube-fed one of four single-substance solutions where the individual drug concentration was matched to the concentration of the corresponding pharmaceutical in the effluent. The drugs selected for single-substance solutions were the four pharmaceuticals with the highest rat dose compared to weight-adjusted DDD. After the fifth and final tube-feeding, several drugs were detected in rat blood serum but in low concentrations. For individual drug compounds, serum concentrations differed between rats tube-fed effluent or single-substance solution highlighting that the absorption and/or, distribution, metabolism and excretion, and in accordance likely the pharmacological activity, of APIs may vary depending on whether they are administered in uncomplicated solutions or in complex mixtures.

The lack of overt responses in the rat exposure study and the clear effects observed in other studies where aquatic organisms have been exposed to the same effluent (Beijer et al. 2013; Carlsson et al. 2009) is likely to a large extent a consequence of the different exposure routes, *i.e.* individual oral exposure versus continuous exposure over the gills. Also, many pharmaceuticals, in addition other chemicals

have the potential to bioconcentrate efficiently from water to fish (Brown et al. 2007; Fick et al. 2010). The fairly low pharmaceutical concentrations in rat serum after five days exposure and lack of obvious effects in rats after oral effluent exposure indicates that acute direct risks associated with drinking of the effluent is low. To reach DDD of any of the measured APIs in the effluent the volume ingested by man needs to exceed 7 L a day. However, for animals which are also more likely to consume contaminated surface water directly, the volume may be significantly lower. Fick et al. (2009) showed that river and lake surface water in addition to well water in the Patancheru region is contaminated with drugs which suggests that organisms, including humans, over a large area could be subjected to prolonged exposures. Also, during low-flow conditions or for particularly sensitive population subgroups concerns may be raised also for short-term exposures. Based on smell alone, the effluent is highly unfit for consumption but the most significant adverse effects for human health may rather be related to indirect risks coupled to potential promotion and spread of antibiotic resistance as a consequence of the environmental pollution of pharmaceuticals.

Antibiotic resistance

Metagenome sequencing

In paper 3 the metagenome from river sediment sampled up- and downstream from PETL was sequenced using pyrosequencing. As a reference, sediment was also sampled up- and downstream from a Swedish sewage treatment plant without any input from drug manufacturing. High abundances of resistance genes for several antibiotic classes including sulfonamides, fluoroquinolone and aminoglycosides were detected in Indian sediment samples. The most abundant antibiotic resistance gene detected was *sul2,* encoding a sulfonamide-resistant variant of an enzyme essential for folate synthesis (Sköld 2000) and the *strA*/*strB* genes, which can inhibit streptomycin by phosphorylating the antibiotic (Ramirez and Tolmasky 2010). These three genes were more abundant at sites downstream from PETL than at upstream sites. Also, the quinolone-resistance genes *qnrD*, *qnrVC* and *qnrS* were detected in the Indian sediment. However, in contrast to the previous resistance genes, *qnr* were overrepresented at upstream Indian sites. Additionally, almost 43% of the identified genes with a pentapeptide repeat

protein structure could not be matched to previously described *qnr* genes suggesting that additional novel *qnr* genes are yet to be identified. None of the above mentioned antibiotic resistance genes were detected in Swedish sediment samples using the pyrosequencing metagenome sequencing technique.

In addition to an overrepresentation of several antibiotic resistance genes in Indian sediment compared to Swedish samples, the relative abundance of a class 1 integrase and a class 2 insertion sequence common region (ISCR) transposase was significantly higher downstream from PETL compared to upstream sites. Transposase enzymes can facilitate the movement of genetic material within genomes or between bacterial chromosomes and plasmids and members from the class 2 family of ISCR transposons have the potential to relocate large segments of DNA including resistance genes (Bennett 2008; Toleman et al. 2006). Moreover, two plasmids (RSF1010 and pMTSm3) were found in high abundance at PETL downstream sites. Both plasmids carry *sul2* and the RSF1010 plasmid also harbor *strA/strB* while pMTSm3 carries a class 2 ISCR transposase (Carattoli 2009; Toleman et al. 2007). Additionally, two novel plasmids could be assembled from the metagenome sequencing data; the plasmid found in sediments sampled upstream from PETL (pHIRE-U1) contained *qnrD*, a fluoroquinolone resistance gene which, at the time, had only been detected once in another genetic context in China (Cavaco et al. 2009). The plasmid pHIRE-D1 detected in Indian downstream samples contained a *sul2* resistance gene in combination with a class 2 ISCR transposase in a similar structure as in pMTSm3. The four plasmids were highly abundant (together accounting for over 4% of total reads in sediments sampled downstream from PETL) which could explain the high detection of *sul2* and *qnrD*. No sulfonamides were detected in a wide screening of sediment why the notably high *sul2* abundance may be a result of co-selection.

qPCR of resistance genes

The exploratory shot-gun sequencing of sediment in paper 3 encouraged a subsequent focused examination of a selection of resistance factors through qPCR. In comparison to the pyrosequencing approach, qPCR lacks exploratory power, but is a more quantitative measurement technique with a substantially higher sensitivity.

Sediment

The qPCR results from Indian river sediments, sampled approximately two years before PETL started to re-route their effluent to another river system, show that sites downstream from PETL is highly similar regarding *qnr* gene levels and composition. The *qnrB*, *qnrD*, *qnrS* and *qnrVC* genes were found in all sediments sampled at the discharge point and downstream from PETL. The *qnrVC* gene was most abundant at all these sites and exceeded the levels of the other common *qnr* genes with about two orders of magnitude. The *qnrA* gene was somewhat less prevalent but was found in similar levels as *qnrB*, *qnrS* and *qnrD* at downstream sites. However, the same genes were also highly prevalent at Indian upstream sites and *qnrB* and *qnrS* were even more abundant at one of the upstream sites than downstream from PETL.

The highly prevalent *qnrVC* gene was first described in a chromosomal integron in *Vibrio cholera* (Fonseca et al. 2008). Other *qnrVC* alleles have been detected as parts of integrative and conjugative elements (Kim et al. 2010; Kumar and Thomas 2011) or located on plasmids in *Aeromonas punctata* and *Vibrio fluvialis* (Xia et al. 2010; Singh et al. 2012). Several species belonging to *Aeromonas* and *Vibrio* genera are common organisms in fresh and brackish water and the plasmid-carrying *A. punctata* was found in wastewater near a Chinese hospital (Xia et al. 2010).

The metagenome sequencing of the same sediment samples detected *qnrD* in the highest abundance followed by *qnrVC* and *qnrS*. The sample preparation protocols for pyrosequencing require more DNA than for qPCR. Due to a limited proportion of organic material (bacteria) in the sediment samples (mainly consisting of gravel at most sites), together with an apparent chemical contamination, the DNA had to be amplified prior to sequencing (Paper 3). As all current protocols for whole genome amplification can induce amplification bias (Abulencia et al. 2006; Pinard et al. 2006), it is possible that the abundance of small plasmids detected in the Indian sediment metagenomes containing *e.g. qnrD* were overestimated in the previous study. Although the results from sequencing and qPCR in general agree well between the studies, *i.e.* both approaches reveal that *qnr* genes are very common in the contaminated Indian

sediments, there are some differences. In contrast to the metagenome sequencing, the qPCR also detected *qnrB* and *qnrA* in Indian sediments. This discrepancy could either depend on an amplification bias as discussed above, or the increased sensitivity achieved with qPCR compared to pyrosequencing, or a combination of both. Among Swedish sediment samples, only two *qnr* genes were detected (*qnrVC* and *qnrS)* which were found once each, both in the sediment sampled closest downstream from the discharge point. The abundance of *qnrVC* and *qnrS* in the Swedish sample was about 1-4 and 4-5 orders of magnitude lower than in the Indian samples respectively which could be a reflection of the low (or absence of) selection pressure expected by FQs in the Swedish river sediments.

Well water and soil

As fluoroquinolones have been detected in high concentrations in a variety of environmental matrices in the Patancheru area (Larsson et al. 2007; Fick et al. 2009; Paper 3), and because they are strictly synthetic and comparably stable substances, fluoroquinolones were chosen to serve as a proxy for a more general contamination of the village well water and soil samples. The s*ul2, intI1* and *qnr* genes were chosen for further analysis as they were all found in high abundances in river sediments downstream from PETL (Paper 3). In addition, *qnr* genes provide specific resistance to quinolones and the *intI1*-associated class 1 integrons may contain several resistance genes and be selected for by a wider range of antibiotics.

In the 2011 January samples, *qnr* genes were detected in 8 of 11 (73%) of well water samples while only 3 of 15 (20%) samples contained detectable *qnr* in June. The *qnrVC* gene was most prevalent and abundant followed by *qnrD* and *qnrB* which was found in three and one village respectively in January water samples. Neither *qnrA,* nor *qnrC* were detected in any samples. The *qnrVC* gene was the only *qnr* detected in soil, in two of the June samples. No support could be found for the hypothesis that *qnr* genes would be more abundant in FQ-contaminated samples. The generally fairly low concentrations of FQ measured in well water and soil at the time of sampling may not be high enough to exert a selective pressure on bacteria carrying the analyzed *qnr* genes, but does not rule out the possibility that other resistance factors are selected for.

The *sul2* and *intI1* genes were highly abundant in both types of environmental samples; 100% hit for *sul2* and 96% for *intI1* respectively. The abundance of *sul2* and *intI1* were significantly higher (p=0.0099 and p=0.0022 respectively) in FQ-contaminated soil sampled in January 2011 and there was a similar tendency (not significant) for well water collected at the same time point. In well water sampled six months later, a weak trend could be seen for *intI1* while *sul2* was significantly more abundant in samples without any FQs detected (p=0.004). In June soil, FQs were detected in two samples only, but in ten times higher concentrations than in January. However, these samples did not show increased levels of neither *sul2* nor *intI1* in comparison to non-contaminated samples. Just prior to the second sampling occasion the yearly monsoon period had begun which may in part explain the somewhat inconclusive data on *sul2* and *intI1* in well water and soil samples which are advised to be interpreted with caution.

Feces

One or more *qnr* gene was detected in 120 of 157 (76%) stool samples from Indian villagers while 9 of 37 (24%) Swedish fecal samples contained *qnr*. The most prevalent and abundant *qnr* genes in Indian stool were *qnrS* and *qnrB* which were both found in over 80% of samples. The same genes were detected in Swedish samples but much less commonly (11% and 8%, respectively). Also, when detected in Swedish stool, the relative abundance of the two *qnr* genes were in most cases about a hundred times lower than in Indian samples, but a few exceptions were found where the levels were similar. The *qnrD* gene was found in 24% of Indian stool samples while detected in four (11%) of the Swedish feces. Although not detectable in some of the Indian villages, the *qnrD* was generally ten times more abundant in Indian samples compared to Swedish stool. None of the other investigated *qnr* genes were found in Swedish fecal samples while occasional hits were detected in the Indian stool.

The considerably higher abundance and prevalence of *qnr* genes in both river sediment and feces from India compared to Swedish samples is consistent with the hypothesis of an original transfer of *qnr* from the environment to human fecal flora in India (Gaze et al. 2013; Wellington et al. 2013). It is by no means easy to pinpoint a site of origin for such transfer events. Indeed, effects on the

composition of the gut microflora could rapidly be blended out as people travel, eat and drink away from home or consume food produced in other areas. For example, during the past years, people in the FQ-polluted villages no longer use well water for drinking (Fick et al. 2009). We have controlled for antibiotic consumption up to six months prior to stool sampling but effects from quinolone treatment could persist for years (Dethlefsen et al. 2008). In addition, even though individuals themselves recently treated with antibiotics were excluded from further analysis, it is possible that consumption by *e.g.* other family members could affect microbial composition as microflora is often easily shared among people living close together (Turnbaugh et al. 2009).

In many of the Indian stool samples, more than one type of *qnr* gene was detected, *e.g.* in 89% of samples *qnrS* and *qnrB* were both found. Almost a quarter of the fecal samples (23%) contained all three of the highly prevalent *qnr* genes (*qnrS*, *qnrB* and *qnrD*). In Swedish stool, only a single sample contained more than one *qnr* gene.

Risks for development and spread of antibiotic resistance in contaminated environments

To assess the risk for resistance development and spread in the environment around PETL (or similar locations), and the potential transfer of resistance to human pathogens, input from many directions is required. A possible starting point might be to identify what resistance-conferring genes and genetic element facilitating their mobility and transfer are present in different environmental matrices. Also the types and concentrations of antibiotics present in the same milieus need to be determined for assessing selection pressures. In paper 3 we started to address these issues and detected a high prevalence of both resistance genes for several classes of antibiotics and an enrichment of elements facilitating gene transfer in river sediments. These results corroborate with earlier findings, suggesting that the environment can be seen as a reservoir from which novel resistance factors may be recruited to human pathogens, and/or where resistance factors already circulating in the human flora may be maintained and recycled. Also, very high concentrations of a specific class of antibiotics (fluoroquinolones) were found in sediment while other possible selective agents, *i.e.* sulfonamide

antibiotics were not found in a broad screening. Earlier reports of FQs in well water in villages located near PETL (Fick et al. 2009) were confirmed in paper 4. Additionally, FQ contamination of soil was documented in several villages in the Patancheru area. In all of these villages, FQs were present also in the well water. The FQ levels in soil were generally modest but suggest an additional potential exposure route of antibiotics into humans as the fields are often used for agriculture and uptake of antibiotics have been documented in crops (Lillenberg et al. 2010; Migliore et al. 2003). The detected concentrations of antibiotics in different environmental matrices in the Patancheru region even far from the PETL effluent discharge point indicates that a large area, and hence an immense number of bacteria, is exposed to antibiotics.

Although raw concentration data on existing selective agents in environmental settings is a valuable starting point, a comprehensive risk assessment should also consider possible additional properties of the environment in question, properties which could potentially influence the bioavailability of the antibiotics. This is likely most relevant for more (semi)-solid matrices where substances may adhere to particles and take part in complex-bindings (Kümmerer 2009), such as in the sediments (Paper 3) and in soil (Paper 4), where the knowledge of what concentrations organisms are actually exposed to is yet limited.

Another key question needed to be further dwelled upon is what exposure concentration is required to exert a selection pressure on resistance determinants, either directly or via co-selection. Significant research efforts are probably needed to answer this query as the minimal selective concentration (Gullberg et al. 2011) likely varies significantly depending on the type of environment, bacterial species, selective medium and resistance mechanism(s) involved (Ashbolt et al. 2013). Gullberg et al. (2011) reported on enrichment of ciprofloxacin resistant mutants after exposure to 100 ng/L of the antibiotic which is in the same concentration range to what was found in some of the well water samples (Paper 4). This data was attained in rigorously controlled competition experiments in the laboratory, however, using only two competing isogenic strains, and the resistance in one of the strains was governed by chromosomal mutations, not by the presence of a *qnr* gene. This represent a highly sensitive system, but it is still an open question what concentrations of ciprofloxacin is required to select for resistant bacteria in a

complex microbial community in the environment. The high prevalence of resistance determinants found in Indian river sediments (Paper 3 and Paper 5) is well in line with the extraordinary high concentration of fluoroquinolones detected. Also, antibiotic concentrations in river water downstream from PETL reached several mg/L levels (Fick et al. 2009), which is clearly selective, *i.e.* exceeds the minimal inhibitory concentration for many bacterial species (EUCAST 2013). As raw sewage containing fecal matter is added to PETL together with the industrial waste, it is difficult to disentangle to what extent the enrichment of resistance factors is due to a selective pressure within the riverbed, inside PETL, or if the genes were highly abundant already in the incoming waste water/sewage. However, the high prevalence of *qnrVC* in sediment but rare detection in stool suggests that may be interpreted as a sign of an ongoing selection in the external environment (PETL/river) rather than a consequence from fecal contamination.

To assess the risk for the dissemination of environmental resistance determinants, information concerning their bacterial hosts and genetic context needs to be obtained. The *intI1* gene was found in high prevalence in Indian river sediment downstream from PETL (Paper 3) and in well water and soil in the Patancheru/Hyderabad area (Paper 4). High levels of the *intI1*-associated class 1 integrons which have the potential to mobilize several classes of resistance genes (Bennett 2008), were recently found in bacterial isolates from inside PETL (Marathe et al. accepted for publication), indicating their broad existence in this environment. Also, qnr genes have been found on integrons (Fonseca et al. 2008; Xia et al. 2010; Kumar and Thomas 2011). The relatively high prevalence of *qnrD* in both Indian sediment and stool samples is somewhat unexpected since the gene was recently discovered in China (Cavaco et al. 2009), and may indicate a wide dissemination of *qnrD* in the area. In paper 3, the *qnrD* gene was found on a novel plasmid which has subsequently been confirmed in European isolates (Guillard et al. 2012; Mazzariol et al. 2012). In Indian fecal samples, qnr genes were commonly co-detected which could indicate that they are located together on more complex genetic elements which could be selected for by FQs or other chemical substances. Also, isolates carrying more than one qnr gene has previously been found (Iabadene et al. 2008; Le et al. 2009; Ferreira et al. 2010; Magesh et

al. 2011) but from current data we cannot determine whether the common finding of several types of qnr genes in the same Indian stool samples is due to the presence of bacteria carrying more than one of these genes. Also, knowledge on what kind of bacteria are harboring the resistance factors detected in the various environmental matrices in the Patancheru area is limited. Other important aspects are whether the bacteria carrying the resistance factor are (common) pathogens, if the carriers readily exchange genetic material with other bacteria (and to whom), and mechanisms which may influence the rate of this transfer. A combination of culture-based experiments and cultivation-independent techniques, metagenome sequencing with an improved depth compared to the previous 454 pyrosequencing approach, may aid the identification of bacterial hosts and elucidate the genetic context of detected resistance genes.

Potential effects on human health caused by antibiotic resistance development and spread are closely linked to the risk for human exposure for bacteria carrying the resistant factors. The effluent generated at PETL was until a few years ago discharged into the local river system offering a potential route for a rapid and widespread dissemination of resistance factors and resistant bacteria. Indeed, resistance genes and mobility factors were highly abundant in river sediments even 17 km downstream from the effluent discharge point route. In paper 5, the possible selection pressure on human fecal bacteria exerted by the FQs detected in well water and soil in was investigated but no enrichment of *qnr* genes were found in stool samples from humans living in FQ-contaminated villages compared to villages where no FQs were detected. This suggests that the current concentrations of FQs in well water and soil were not causing a transfer of *qnr* genes from the environment to the human gut. However, it is of course possible that the *qnr* genes are following the selection patterns of other selective agents not studied here. Also, albeit from a limited number of samples, data suggests that the FQ concentration in well water in the Patancheru area is decreasing (Fick et al. 2009; Paper 4) possibly as a consequence from PETL rerouting effluent to another waste water treatment plant, initiating in July 2009 (APPCB 2010). As the environmental FQ levels may have been higher, and hence the selection pressure stronger, prior to the first stool sampling (July 2010), it cannot be excluded that *qnr* gene acquisition and maintenance in environmental bacteria and even transfer

to human microflora was more strongly promoted at an earlier time point. Furthermore, in addition to the hypothesis of a continuous transfer of resistance factors or resistant bacteria from the environment to human gut it is also possible that this transfer is a rare or even a one-time event and the major dissemination of resistance occur via the transmission between individuals (Ashbolt et al. 2013). If so, the initial "village contamination effect" may quickly become undetectable as resistance can rapidly spread from the site of emergence (Harris et al. 2010; Walsh et al. 2011). To observe an environmental origin of a resistance factor found in human gut flora, field data including stool samples should ideally be collected in time series from both clean and contaminated sites to find epidemiological evidence for what has already occurred with regards to gene prevalence and abundances and/or to increase the likelihood of capturing the early appearance of the resistance before a potential spread in human gut flora.

Once reached the human body, to have any significant impact on our health, the resistance-carrying bacteria must also be able to colonize in the new environment and/or share their resistance traits with other already established bacteria. Both of these processes may be promoted by exposure to a selective agent, *e.g.* an antibiotic substance, which could offer selective advantages to the resistance-carrying bacteria. We speculate that the observed international differences in *qnr* prevalence and abundance in stool samples are, at least in part, a consequence of the lower and more strictly controlled usage of FQs in Sweden compared to India where FQ are generally inexpensive and sold over the counter without the need for a prescription. The use, overuse and misuse of fluoroquinolones in the Indian community may have contributed to a general selective pressure advantageous to bacteria less susceptible to this type of antibiotic substances.

To summarize, the high antibiotic-contamination of river sediments around PETL have enriched resistance determinants corresponding to several classes of clinically relevant antibiotics and genetic elements facilitating their mobility. The sediments may thus act as a reservoir from which resistance traits could be spread with the potential to ultimately reach human pathogens. In the well water and soil where the measured antibiotic concentrations are lower than in sediment samples, no clear indication of an enrichment of resistance factors in contaminated environments was found at the current concentrations of FQs.

However, it is possible that *qnr*-carrying bacteria have had a selective advantage in the Indian community when the environmental concentrations of fluoroquinolones were higher (Fick et al. 2009), *e.g.* prior to PETL effluent rerouting. We found no support for the hypothesis that a selection pressure from FQs plays a crucial role in the acquiring of *qnr* genes for bacteria found in Indian stool samples. It is difficult to estimate how long *qnr* genes have been present in human gut flora first but we know that they have been present in clinical isolates for at least 15 years (Martinez-Martinez et al. 1998). Hence, it might have been too late to make any links to a specific environment or area (Ashbolt et al. 2013). As we have focused on analysis of a narrow range of both antibiotic compounds and resistance genes it cannot be excluded that other selective agents are indeed present in these matrices in concentrations which exert a selective pressure on relevant resistance determinants not investigated here.

Findings of high environmental levels of pharmaceuticals

For the past decades, significant environmental discharges from drug manufacturing have been reported from different parts of the world, including Europe (see table 4). In the early 90's, over 2g/L of salicylic acid was found downstream from a production facility in India (Bisarya and Patil 1993). A few years later, mg/L levels of sulfonamides could be detected in the groundwater adjacent to a closed landfill previously used for pharmaceutical waste disposal in Denmark (Holm et al. 1995). Dumping of pharmaceutical waste off the coast of Puerto Rico in the 70's and 80's was shown to affect the number, composition and metabolic activity of bacterial species in the surface water and waste plume (Peele et al. 1981). In China, ethinylestradiol in effluent from a contraceptive factory wastewater plant was detected in 50ng/L levels (Cui et al. 2006), which is more than 10 times higher than the concentrations known to cause divergence from normal sexual differentiation in fish (Purdom et al. 1994; Länge et al. 2001). Another Chinese study found almost 20 mg/L of oxytetracycline in the treated wastewater from a manufacturing site and the antibiotic could be detected in the receiving river about 20 km downstream from the discharge point, albeit at approximately 50 times lower concentration (Li et al. 2008). Water grab samples near drug formulation facilities in Pakistan contained >1µg/L concentrations of

several antibiotics (Khan et al. 2013). Sim et al (2011) reported on over 40 mg/L of the antibiotic lincomycin in treated effluent from pharmaceutical industries in South Chorea. In addition to reports from Asia, when effluents from treatment plants in New York were screened for opioids and muscle relaxants, the levels were generally <1µg/L except in plants which had also received wastewater from pharmaceutical production, where *e.g.* the oxycodone was found in mg/L levels (Phillips et al. 2010). There are also examples of releases of antibiotics (Babic et al. 2007; Thomas et al. 2007); analgesics, (Reddersen et al. 2002; Zühlke et al. 2004) and antivirals (Prasse et al. 2010) from European drug manufacturers.

Table 3. Examples of environmental releases of active pharmaceutical ingredients from drug manufacturing.

Pharmaceutical detected	Country	Reference
Salicylic acid	India	Bisarya *et al.* 1993
Sulfonamides	Denmark	Holm *et al.* 1995
Phenazone	Germany	Zülhke *et al.* 2004
Fluoroquinolones	India	Larsson *et al.* 2007
Ethinylestradiol	China	Cui *et al.* 2006
Bacitracin	Norway	Thomas *et al.* 2007
Sulfonamide antibiotics	Croatia	Babic *et al.* 2008
Oxytetracycline	China	Li *et al.* 2008
Fluoroquinolones	India	Fick *et al.* 2009
Oseltamivir	Switzerland	Prasse *et al.* 2010
Oxycodone	USA	Phillips *et al.* 2010
Lincomycin	Korea	Sim *et al.* 2011
Antibiotics	Pakistan	Khan *et al.* 2013

To contrast the numerous findings of high environmental concentrations of drugs from production facilities, to my best knowledge, a single study exists reporting of only minor discharges (in the low µg/L range) of pharmaceuticals in waste water

from pharmaceutical industries (Taylor 2010). Though, it should be acknowledged that it may be a hard undertaking getting “negative” results published which may explain the highly limited data on low levels of APIs from drug manufacturers. The high environmental concentrations of drugs from pharmaceutical production detected in Patancheru are thus not an isolated event. Neither is this type of findings limited to specific geographical regions but wide-spread phenomena occurring in many parts of the world.

The pharmaceuticals produced in the Patancheru region are to a large extent intended for the global market and many of them end up in Swedish pharmacies (Larsson and Fick 2009). This infers that the Western world share the responsibility for the pollution of environments even far away from home. When it comes to antibiotic resistance it seems to matter very little where it first emerges, our travelling habits, medical tourism, import and export practices etc. can cause rapid and extensive geographical dissemination of resistance genes, as recently observed with the New Delhi metallo-beta-lactamase (NDM-1) discovered in India in 2009 (Yong et al. 2009; Walsh et al. 2011).

Future perspectives

The persistence of many pharmaceutical compounds and the extensive, continuous and growing use of both human and veterinary drugs worldwide suggests that the emissions of and effects from residual drugs, including antibiotics, in the environment will remain a challenge also in the near future. Rather than a complete picture of the mechanisms behind, and factors influencing the recruitment, maintenance and dissemination of resistance determinants and/or resistant bacteria, it is evident that we are currently stuck with a giant jigsaw puzzle where several significant pieces of information are still missing. However, if the risks associated with underdimensioned or even absence of actions are considered serious enough, based on the precautionary principle prompt measures may be motivated even though some information is yet lacking. Additionally, while discrete exertions taken at isolated locations or even in individual countries may, at best, lead to positive but potentially transient and demarcated effects, international collaboration efforts combining further research and strategies for better waste management is likely required to reach sustained effects on a global scale.

Proposed focus points for future research

- Monitoring of pharmaceutical concentrations in waste from drug manufacturing to establish the proportions of the problem. Surveillance should take place worldwide as significant discharges from pharmaceutical production also in industrialized countries cannot be excluded.
- Monitoring of patterns and fluctuations in the prevalence of antibiotic resistant bacteria and resistance determinants, both in clinical and environmental settings. Data from separate input sites should be combined so potential trends in *e.g.* resistance rates on a national or even global scale can be spotted early to possibly hinder the spread of new resistance factors.
- Expansion of the minimal selective concentration concept (Gullberg et al. 2011), *i.e.* determining the lowest concentration of selective agent where

the potential fitness costs associated with the acquiring of the resistance factor are counterbalanced by the benefits from the new trait and bacteria are selected for. Additional studies from various environmental matrices could provide data to establish these thresholds of antibiotic concentrations in natural and complex bacterial ecosystems.

- Further investigations on what factors are influencing mutation frequency and likelihood for HGT among bacteria. This may include additional studies in "hotspots", *e.g.* WWTPs, which have been described as ideal milieus for HGT (Schlüter et al. 2007), and comparisons to other more pristine environments.
- Deeper understanding of the genetic context of resistance determinants, *e.g.* exploring their potential location on genetic elements enabling their mobility and the pathogenicity of the bacterial hosts. A combination of culture-based and cultivation-independent techniques is likely required to investigate this.
- Exploration of the potential link between the price of a pharmaceutical and the pollution control in the production chain. Is it "safer" for the environment to buy more expensive products, or not? This has been speculated on, but there is as yet no evidence for this.
- Investigate whether reformed pharmaceutical pricing systems can create incentives for decreased emissions from drug production. A key question to consider is whether any potential cost increases resulting from a more environmental-friendly manufacturing should be financed by individual costumers/patients or governmentally funded through *e.g.* reimbursement systems (Swedish Government, 2013). An assessment system is required to be able to classify environmental risks associated with different manufacturing approaches.

Improved management of discharges from drug production

- Define emission limits for releases of active pharmaceutical ingredients from production facilities. Such limits are currently rare. Accordingly,

manufacturers must prove that discharges are kept below agreed restriction limits.

- Consider the whole life-cycle of pharmaceuticals in environmental risk assessments. Currently, the predicted environmental concentration of new pharmaceuticals to be introduced to the EU and US market is based solely on releases from usage but does not take into account emission data from potential discharges during drug manufacturing (FDA 1998; EMEA 2006).
- Improve/amend treatment techniques in WWTPs based on contents of influent. For example, Pruden et al. (2013) recommend that activated sludge treatment of waste water containing significant amount of antibiotic substances are avoided, or, if this technique is used, that the management of resulting wastes is improved, *e.g.* monitoring of the levels of antibiotic resistant bacteria and resistance genes in resulting before land-application. Also, the results of chosen actions should be monitored to quantify the relative significance of various management efforts which could in turn be used to direct future measures.
- Include environmental criteria in the good manufacturing practice framework for pharmaceutical industries. This was the main suggestion from the Swedish MPA after being commissioned by the Swedish government to investigate (Swedish MPA 2009; 2011) and has been brought to the health ministry of EU.
- Improve the transparency of the pharmaceutical production chain (Larsson and Fick 2009; Larsson 2010). As of today, information on where and under what conditions drugs are manufactured is confidential. If the specific origin of the API for each product is made publically available, it is likely that the companies selling the final product would increase their efforts to reduce risks for negative headlines in media by working harder with their manufacturing chains. Thus, potential pressure from final consumers may provide incentives for manufacturers to invest in improved, but not initially cost-effective, waste treatment technologies.

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