Effects of pharmaceuticals on natural microbial communities

Tolerance development, mixture toxicity and synergistic interactions

SARA BROSCHÉ, 2010

UNIVERSITY OF
GOTHENBURG

FACULTY OF SCIENCE

DEPARTMENT OF PLANT AND ENVIRONMENTAL SCIENCES

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Examinator: Professor Göran Dave, Institutionen för växt- och miljövetenskaper, Göteborgs universitet

Fakultetsopponent: Dr Alistair Boxall, Environment Department, University of York, Great Britain

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Abstract

Due to our extensive use of pharmaceuticals, low concentrations (picomol-nanomol/L) end up in the aquatic environment. Antibiotics comprise a group of pharmaceuticals specifically designed to disrupt microbial biochemical processes, and might therefore in particular have detrimental effects on microbial communities in the environment. However, current environmental risk assessment strategies of pharmaceuticals do not necessarily suffice for protecting environmental microbes. Therefore, the ecotoxicity of pharmaceuticals were assessed on natural bacterial communities to provide ecologically more realistic data and to improve the knowledge about their environmental hazard.

Paper I, III and IV in the thesis focussed on the effects of antibiotics. It was shown that in particular chlortetracycline, but potentially also ciprofloxacin, is clearly toxic already at concentrations currently detected in the environment, hence posing an environmental risk to environmental bacteria. In paper II, attached microbial communities were exposed to 5 pharmaceuticals and personal-care products (PPCPs) (fluoxetine, propranolol, triclosan, zinc-pyrithione and clotrimazole), which all showed to be toxic towards the algae, however only at concentrations below currently detected.

Many pharmaceuticals are often simultaneously present in sewage treatment plant effluents. Hence, the exposed microbial communities in the recipient are subjected to a mixture of active substances. Mixtures do generally cause higher effects than each of their comprising substances alone, and it is therefore also important to consider also their combined toxicity. Based on the experimentally determined effects of the individual substances, two mathematical concepts have been suggested for predicting toxicity of mixtures comprised of similarly and dissimilarly acting substances: Concentration Addition (CA) and Independent Action (IA). Their applicability is generally accepted for single species assays, and the results in paper I and II in the thesis supports their validity also at a community level of biological complexity. However, both concepts are based on the assumption that no interactions occur between the mixture components.

One such interaction would be the effect of chemosensitizing substances that inhibit bacterial efflux of antibiotics, thus increasing their toxicity beyond the predicted. Therefore, the combined effects of 3 proven chemosensitizers and the antibiotic ciprofloxacin on natural bacterial communities were investigated in paper IV. As opposed to results from clinical studies, no increased effects beyond what was predictable by IA were seen. Chemosensitization seems therefore be of low importance in natural bacterial communities.

Poorly controlled pharmaceutical production facilities have recently been shown to release extremely high amounts antibiotics. Apart from the high toxicity of this pollution, concerns were raised with respect to bacterial resistance development in the receiving river. Therefore, the potential for tolerance development in microbial communities were assessed in paper III, using either treated effluent from an Indian production site or ciprofloxacin at corresponding concentrations. Both exposures induced tolerance of the bacterial communities towards ciprofloxacin, the effluent to the highest extent. However, whether this was due to resistance development or not needs to be further investigated.

To conclude, this thesis shows that current environmental hazard assessment strategies for pharmaceuticals and antibiotics might not be realistic enough to protect natural microbial communities, and should therefore be extended accordingly. The results also emphasize the need to take complex environmental exposure situations into account, and to especially consider the combined toxicity of pharmalceuticals in the environment.

Keywords: antibiotics, pharmaceutical mixtures, microbial community ecotoxicology, Concentration Addition, Independent Action, community tolerance development, chemosensitization

I reject your reality

and substitute my own.

Adam Savage, Mythbusters

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This thesis is based on the following papers:

I. Brosche, S., Backhaus, T., 2010. *Toxicity of five protein synthesis inhibiting antibiotics and their mixture to limnic bacterial communities*. Aquat. Toxicol. 99, 457-465.

II. Backhaus, T., Porsbring, T., Arrhenius, Å., Brosche, S., Johansson, P., Blanck, H. *Single substance and mixture toxicity of 5 pharmaceuticals and personal care products to marine periphyton communities*, submitted to Environmental Toxicology and Chemistry

III. Brosche, S., Fick, J., Larsson, D.G.J., Backhaus, T. *Effluent from antibiotic production induce tolerance development in natural freshwater bacterial communities*, submitted to FEMS microbiology Ecology

IV. Brosche, S., Backhaus, T., *Effects of chemosensitizers on the uptake and toxicity of ciprofloxacin in natural bacterial communities*. Manuscript.

Some abbreviations commonly used in the thesis

- EC = Effect Concentration, e.g. EC50 the concentration needed to provoke 50% effect
- NOEC = No Observed Effect Concentration
- PNEC = Predicted No Effect Concentration
- MIC = minimum inhibitory concentration
- PEC = Predicted Environmental Concentration
- EIC = environmental introduction concentrations
- AWC =Awerage Well Colour
- AUC =Area Under the Curve
- PPCPs = Pharmaceuticals and Personal Care Products
- CA = Concentration Addition
- IA = Independent Action

1 | Pharmaceuticals in the environment

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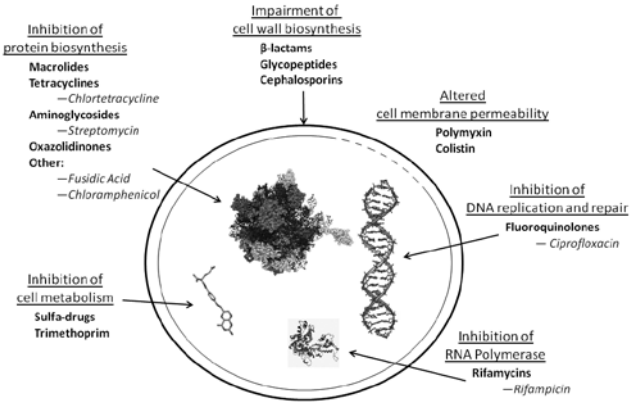
The term pharmaceutical is generally used for a chemically and structurally diverse group of substances used within human and veterinary medicine. Their only common denominator is that they are all designed to interact with different biological pathways, causing a specific physiological response. This is obviously a preferred quality from a medicinal perspective, but might be of concern for non-target organisms when pharmaceuticals are released into the environment.

Pharmaceuticals are grouped according to the organ or system on which they act and/or their therapeutic and chemical characteristics in the Anatomical Therapeutic Chemical (ATC) Classification System, which is controlled by the WHO Collaborating Centre for Drug Statistics Methodology (WHOCC). One such group is the anti-infectives (group J), which encompasses antibacterials, antibiotics, antifungals, antiprotozoans and antivirals. They are different from most other pharmaceutical groups in that they are "licensed to kill", i.e. they are meant to eradicate microbes harmful for e.g. the human body. They are in this sense closely related to the biocides used in personal care products (PCPs), e.g. triclosan and zinc pyrithione investigated in paper II.

Three out of four studies in this thesis have focussed on antibiotics, a pharmaceutical innovation most likely contributing substantially to our increased life expectancy during the last 50 years (Schnittker and Karandinos, 2010). The term antibiotic will throughout this thesis be used for substances that are specifically used to fight bacterial infections, in contrary to e.g. disinfectants and preservatives. Antibiotics act mainly by 5 general modes of action: inhibition of cell wall biosynthesis (e.g. penicillin), impairment of structure and function of the cell membranes (e.g. polymyxin E), inhibition of DNA-biosynthesis and –reproduction (e.g. ciprofloxacin), inhibition of folate synthesis (e.g. sulfamethoxazole) and inhibition of protein biosynthesis (e.g. streptomycin) (Courvalin, 2008). Even though members of the same class share mode of action, they often have different mechanisms of action, i.e. they bind to different targets.



Although the first antibiotic was synthesized already in the early 1900s, it was during the 1940s and 1950s the foundation for the antibiotics of today was laid with the discovery and means of isolating e.g. penicillin, the tetracyclines and macrolide antibiotics. The German scientist Paul Ehrlich coined antibiotics "magic bullets", i.e. substances that specifically could kill bacteria without harming the person infected. Suddenly, bacterial infections previously fatal could be contained and cured. However, almost immediately the flip-side of the coin became evident. Within a few years of introduction, most hospital isolates of *Staphylococcus aureus* were resistant towards penicillin, a possibility Alexander Fleming had already warned about in his 1945 Nobel Prize lecture. Today, clinical resistance has been shown towards all antibiotics available (McDermott, 2003), and the relative ease by which we have come to expect bacterial infections to be cured soon belongs to the past.

A less noticeably side effect of the extensive use of antibiotics was mostly disregarded until the early 1990s, i.e. the release of antibiotics into the environment making the ubiquitous aquatic contaminants (Boxall, 2004).

1.1 Use

Pharmaceuticals are mainly used within human and veterinary medicine. However, the general efficacy of antibiotics to fight bacterial infections has widened their use to include also agricultural applications to prevent crop damage. They are also used as feed additives for live stock, i.e. as so-called growth promoters, in order to eliminate bacteria in the gut hence both decreasing the competition for nutrients and reducing microbial metabolites that might depress growth. Both these additional uses have been banned within the European Union since 2006 (with Sweden as forerunner, banning them already in 1986) due to the spread of resistant bacteria, but is still allowed e.g. in the US (Dibner and Richards, 2005; Kummerer, 2009c).

In Sweden, roughly 10 000 pharmaceutical products are approved today. Total pharmaceutical sales have increased almost linearly the past 25 years, amounting to 4 billion Euros in 2009 (0.6% of the global sales of 630 billion Euros). The same year, Swedish sales of anti-infectives amounted to about 300 million Euros (LIF, 2010). When it comes to antibiotics, about 64.5 tonnes of human antibiotics were sold in Sweden in 2009 and 14.7 tonnes for veterinary purposes (SWEDRES, 2009). Data on international sales is usually not publicly available and therefore comparisons are problematic, but it was estimated that the word wide market consumes 100,000 – 200,000 tonnes of antibiotics every year (Wise, 2002). In the US, 16 thousand tonnes of antibiotics were used annually in the beginning of the millennium (Sarmah et al., 2006) and 5400 tonnes was used only in veterinary medicine within the European Union in 2004 (Kools et al., 2008).

1.2 Routes into the environment

After application most pharmaceuticals are metabolized to some degree in the body, the extent depending on the chemical properties of the drug, e.g. of the antibiotic amoxicillin 80–90% is excreted unchanged (Bound and Voulvoulis, 2004). Therefore, a certain amount of the active substance will be excreted together with more or less active metabolites, enter the sewage system and finally end up in a sewage treatment plant (STP). A small contribution to the overall load into the sewage system is also unused drugs that are improperly disposed of (Daughton and Ruhoy, 2009). Hospital waste waters constitute a special case, where generally higher concentrations of pharmaceuticals are detected (Lindberg et al., 2004; Martins et al., 2008). They can either be connected to the municipal STP or have a separate hospital STP, the latter not necessarily ensuring higher removal rates of the pharmaceuticals (Kosma et al., 2010) .

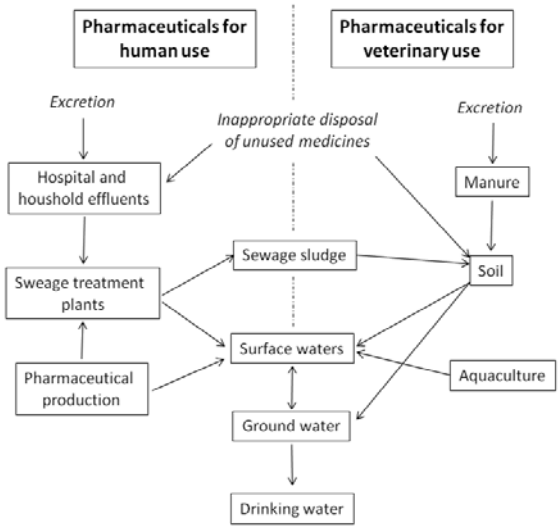


Figure 2 An overview of the routes by which pharmaceuticals enter into the environment

Municipal STPs are not designed to remove antibiotics or other pharmaceuticals, but to limit the release of nutrients and organic matter into the aquatic environment. Even so, some pharmaceuticals are removed during the treatment process due to adsorption, photolysis and bacterial degradation. However, due to the chemical properties of the pharmaceutical removal can differ quite substantially, e.g. the β-blocker atenolol is not removed at all, whereas paracetamol is removed almost completely (Miege et al., 2009). In the common case the treated sewage effluent is released by the STP into a nearby river, still containing small amounts of pharmaceuticals. When pharmaceuticals are used within veterinary medicine, the ingested drug will be excreted directly e.g. onto a pasture, potentially being flushed into nearby streams during rain fall.

It was recently shown that also the production of pharmaceuticals can lead to environmental contamination when insufficiently controlled. Extreme concentrations of oxytetracycline (43 µmol/L) in STP effluents connected to production facilities in China was reported by Li and colleagues (Li et al., 2008), and Larsson and co-workers reported a total concentration of fluoroquinolone antibiotics of 100 µmol/L in effluent from drug production facilities in India (Larsson et al., 2007). These amounts are in the same range as the human serum concentration during treatment.

1.3 Occurrence in the environment

A majority of all analytical studies on pharmaceuticals in the environment focus on concentrations detected in STP effluents and surface waters. However, pharmaceuticals have been detected in all aquatic compartments even if the comparative knowledge on their presence in groundwater, drinking

water and sea water is low. The detection of pharmaceutical substances is not a measure of the number actually present, since most studies do not have the aim to determine all, but are targeting a certain group.

Pharmaceuticals from all therapeutic groups have been detected in STP effluents, mainly in the pico-nmol/L concentration range. Highest concentrations are generally found by for high volume drugs, e.g. anti-inflammatory drugs.

As a consequence, the highest environmental concentrations are found in surface waters, see e.g. (Coetsier et al., 2009). Still, the groundwater concentrations of the antiepileptic drug carbamazepine have been detected up to 5 nmol/L (Heberer, 2002). E.g. the presence of clofibric acid, propylphenazone and diclofenac was determined in the drinking water of Berlin in the nmol/l concentration range (Khetan and Collins, 2007). Due to e.g. the leaching behaviour of antibiotics applied in veterinary medicine, sulfa-antibiotics have been detected in groundwaters (Blackwell et al., 2009), however it should be noted that antibiotics still have not been detected in drinking waters (Kummerer, 2009b). Finally, e.g. salicylic acid at 5 nmol/L was detected in the marine environment (Wille et al., 2010).

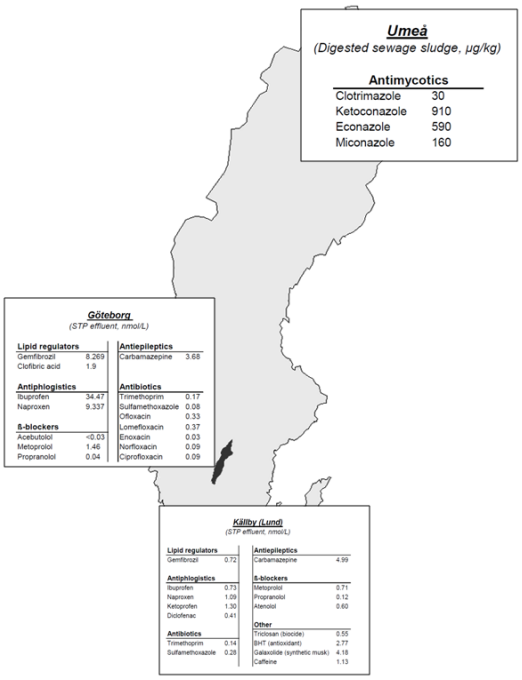


Figure 3 Examples of pharmaceuticals detected in Swedish sewage treatment plants. Data collected from (Andreozzi et al., 2003; Bendz et al., 2005; Lindberg et al., 2010)

When it comes to environmental detection data on antibiotics, a certain background concentration can be expected in soil, since several of the "natural" antibiotics are produced by soil living organisms, e.g. streptomycin by the bacterium Streptomycetes. However, no such production has been showed for the aquatic environment so far (Kummerer, 2009a), which means all measurable concentrations detected there are most likely introduced through human use.

2 | Ecotoxicology of pharmaceuticals

Each aquatic environment constitutes a complex ecosystem of its own, where physicochemical properties combined with the dynamic interactions between individuals, populations and communities define its structure and function. Hence, the presence of a pharmaceutical that primarily affects one species might cause indirect effects on the whole ecosystem by affecting this balance.

Effects of pharmaceuticals in the aquatic environment have been studied at all levels of biological complexity, e.g. for synthetic estrogens ranging from whole lake manipulations (Pelley, 2003) to gene expression in individually exposed fish (Corcoran et al., 2010). While the former level is (for obvious reasons) not a practical solution for all studies, the latter will ignore all effects beyond ones on the individual, in addition population, community and ecosystem effects will be missed.

Therefore, natural microbial communities have been established as a convenient, yet environmentally relevant, level of organisation to study. Microbe is a broad term used for organisms best studied or only seen with a microscope, meaning bacteria, fungi, microalgae, protozoa, and viruses. In this context, natural denotes communities established and sampled directly from the environment, as opposed to communities formed under anthropogenic influence, e.g. sewage sludge communities. Environmental microbes generally exist in planktonic form or organised in complex biofilm communities (epi- or periphyton), attaching to any available surface. Periphyton are highly structured entities where a diverse range of species compete for space and available nutrients, each with its own strategy and sensitivity towards different stressors. The generation time of the comprising organisms are comparatively short (hours-days), and therefore a continuous succession in the community can be studied within a feasible time-frame but still similar to e.g. the much slower succession of higher plants (Hoagland et al., 1982). Since all commonly studied ecosystem processes can be seen in the periphyton community, e.g. grazing, nutrient cycling and decomposition, they have even been called a micro-ecosystem (Bosserman, 1983). Due to the integrative properties of the periphyton communities, both direct and indirect effects can be captured by assessing effects only on part of the community. Indirect effects typically include changes in the relative abundance of an organism even though not affected by a certain toxicant, e.g. toxic effects on the grazing part of the community may manifest itself as increased algal biomass. Hence, the outcome of an exposure will be an integrated response of all species present. Effects on communities are commonly divided into structural and functional endpoints, i.e. effects on the species composition and abundance of the community and effects on the community performance of a selected function, e.g. photosynthesis or respiration. The endpoints are obviously interconnected, but both kinds should preferably be assessed .

The studies in this thesis focussed on effects measured on the bacterial and algal part of microbial communities. Bacteria are important degraders of organic matter, making degradation and mineralisa-

tion products available as nutrients available for other organisms in the food web (Hofle et al., 2008). Since many bacteria might be highly particular in their substrate preferences (Lowe et al., 1993), and might perform specialized functions both their abundance and diversity are important for maintaining cycling of nutrients within the ecosystem (Reed and Martiny, 2007). Microalgae and cyanobacteria are primary producers (i.e. they produce biomass from inorganic compounds), and therefore form a vital part of the base of the food web. Hence, microbial communities are clearly vital for maintaining the function of the food web.

Pharmaceuticals are generally developed to target a certain biological pathway, in some cases even to act on a specific receptor. This does, however, not imply that they only affect the intended organism or even the same target in a non-target species. It is well known that pharmaceuticals have side effects already in the human body due to binding to different molecular targets than intended. That such receptors are present also in non-target organisms is therefore not hard to imagine, and effects caused by non-target receptor binding cannot be excluded. One example of this is the heart medicine propranolol tested in paper II, which blocks the action of epinephrine and norepinephrine on both β1- and β2-adrenergic receptors in the human body (Mehvar and Brocks, 2001). Green algae have no adrenergic system and hence lack the same receptors, but still propranolol is toxic to green algae. Not only is there a target site for the drug in the algae, the outcome of binding is fundamentally different from the mechanism of action in the human body. Thus, absence of the intended drug target does not mean absence of effects on an organism.

Most pharmaceuticals have a very specific mode of action compared to common industrial pollutants such as organic solvents. Hence, effects at the molecular target site can generally be seen after only a short time period. However, effects on more integral endpoints such as photosynthesis or growth inhibition generally take longer to manifest themselves. Therefore, acute toxicity of pharmaceuticals must be assessed using endpoints closely related to the activity of the drug in order to obtain a relevant estimation of effects, whereas chronic studies are better suited e.g. for studying growth. The time issue is especially pronounced when it comes to antibiotics, since they are in general bacteriostatic than bacteriocidal, i.e. they inhibit reproduction of the bacteria rather than killing them. This can be exemplified by the more than 1000-fold lower EC50 of the antibiotic chloramphenicol after 24h in the bioluminescence inhibition assay compared to the EC50 after 30 minutes of incubation (Froehner et al., 2000).

Effects of pharmaceuticals on bacteria and microalgae have been extensively investigated in standardized single species assays, which generally confirm the low acute toxicity. When e.g. determining acute toxicity (30 min) in the bioluminescence inhibition test with the marine bacterium *V. Fischeri*, EC50s were only seen at concentrations far removed from any ecologically relevant, i.e. in the upper µmol/L or mmol/L range for e.g. antibiotics (isidori 2005, lalumera 2004), beta-blockers (Escher 2006 ES&T) and anti-inflammatory drugs (Farré 2001). However, chronic toxicity of two substances studied in paper II have been seen at environmentally relevant concentrations: 40% reduction of sterol content in periphyton communities was seen at 0.5 nmol/L of clotrimazole (Porsbring et al., 2009), and a growth inhibition EC50 of 1.8 nmol/L when exposing the micro algae *P. subcapitata* to the general biocide triclosan (Yang et al., 2008).

2.1 Bacterial resistance / community tolerance in natural bacterial communities

Microbes respond quickly to changes in their environment, and a microbial community is a dynamic entity in a state of constant succession (Jackson, 2003). Since different species within the community inherently have different sensitivities towards different stressors, e.g. some bacteria cope better with cold temperatures, whereas other might have a high resilience towards shifts in nutrient availability. No species is consistently more sensitive towards all stressors, e.g. the sensitivity of microalgae towards different toxicants has been shown to differ by several orders of magnitude (Blanck et al., 1984). When it comes to antimicrobial substances like antibiotics, they are well known to have limitations in their activity spectrum, i.e. they might only target either gram positive or gram negative species. Hence, when a microbial community is exposed to a toxic stress, the sensitive individuals will be replaced by more tolerant ones and a toxicant induced succession will occur. This will result in a generally more tolerant community, a process referred to as Pollution Induced Community Tolerance (PICT) which can be due either to structural changes in the community (changes in species composition) or biochemical changes in the species present rendering them more tolerant, i.e. genetic changes or physiological adaptations (Blanck, 2002; Blanck and Wangberg, 1988). The latter has been extensively investigated for antibiotic resistance development in single strains of human pathogens, but is still not fully understood (Martinez et al., 2007). It is however clear that clinical resistance evolves in response to an antibiotic exposure, selecting for the individuals with genotypes coding for a slightly less sensitive phenotype (Davidson and Surette, 2008). This process can occur quickly at high concentrations of antibiotics, since bacterial populations adapt rapidly to environment changes (Elena and Lenski, 2003; Perron et al., 2008).

The major resistance strategies utilized by bacteria are shown in fig 4. They can either be chromosomally or plasmid encoded (Fajardo et al., 2008), the former being remnants of ancient bacterial pathways proposed to originally been part of e.g. metabolic processes (Martinez, 2009). Seen from an evolutionary perspective, plasmid encoded resistance is mainly a recent development, greatly multiplied after the introduction of antibiotic therapy in the 1940s (Aminov and Mackie, 2007; Knapp et al., 2010). Therefore, the general efflux mechanisms conferring resistance to a wide variety of substances are generally chromosomally encoded, whereas efflux mediated resistance to specific antibiotics are plasmid encoded.

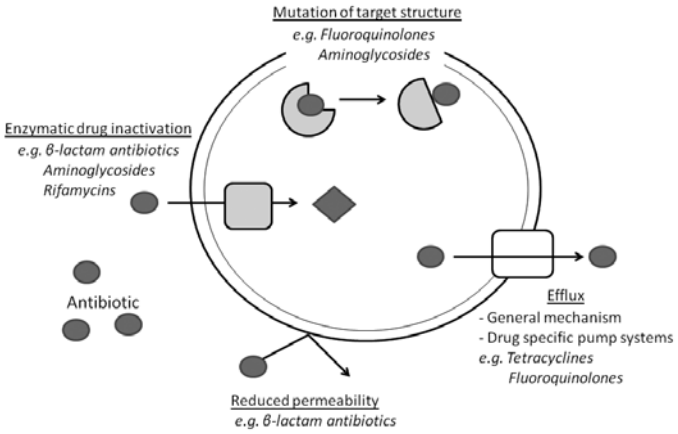


Figure 4 Common resistance baterial mechanism to antibiotics

Several mechanisms induced by antibiotic exposure will further increase the evolution of antibiotic resistance. Antibiotics have been shown to induce mutagenesis (Galhardo et al., 2007), and mutations conferring antibiotic resistance will be beneficial and selected for (Martinez and Baquero, 2000). Also,

antibiotics induce the SOS response systems in bacteria, which increase mutation rates, activates mobilization of many mobile elements (Fajardo and Martinez, 2008; Martinez et al., 2007) and increase rate of gene transfer that result in antibiotic resistance (Aminov, 2009). For a more in depth discussion on these mechanisms, see e.g. the review by (Couce and Blazquez, 2009) and references therein.

Sewage treatment plant effluents have been suggested as potential hotspots for resistance development of environmental bacteria (Goni-Urriza et al., 2000), especially when containing high concentrations of antibiotics (Li et al., 2010). Resistance genes for all major antibiotic groups have been detected in STPs and their receiving waters (Zhang et al., 2009). Therefore, STPs in general have been suggested as reactors for resistance development. However, the origin of these resistance genes is not necessarily bacteria from the STP, since both resistance genes and resistant bacteria are part of the incoming raw sewage (Baquero et al., 2008). Nevertheless, an increase in the instance of resistant bacteria has been shown downstream sewage treatment plants (Kim and Aga, 2007). Therefore, attached microbial communities in STP effluent recipients provide a platform for environmental bacteria to mix with resistant bacteria transported by the effluent. The high bacterial density and high metabolic activity in the biofilm make them uniquely suited for exchange of genetic material such as resistance elements (Sorensen et al., 2005). Hence, biofilms have been labelled one of the main "genetic reactors" supporting and proliferating resistance development in the environment (Baquero et al., 2008).

3 | Regulatory environmental risk assessment

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The European Union and the US together comprise 70% of the global pharmaceutical market(Sweden, 2010). I will therefore give a brief outline of their regulatory risk assessment strategies, focussing on the same area as the thesis, i.e. the aquatic environment.

3.1 Pharmaceuticals for human use

Before putting a new human pharmaceutical substance on the market, an environmental risk assessment (ERA) is required. Within the EU, this is regulated by the Guideline on the environmental risk assessment of medicinal products for human use (REF) issued by the European Medicines Agency (EMA). In the US, the Food and Drug Administration (USFDA) is required to assess possible environmental effects as a part of the regulatory process of approving a new drug. The detailed steps are defined in the guideline on Environmental Assessment of Human Drug and Biologics Applications.

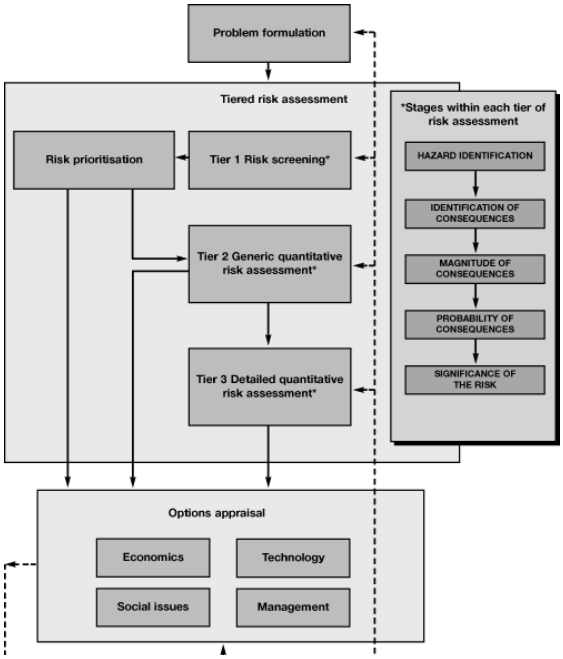

Figure 5 An overview of the tiered approach of environmental risk assessment. From Department for Environment, Food and Rural Affairs (Defra), the UK.

The different phases/tiers of the assessment are called differently in the European and US guidelines, but are based on principally similar approaches. Both guidelines start with a Tier 0, which is based on a worst case estimation of environmental concentrations. The European guideline is based on the estimation of Predicted Environmental Concentrations (PECs), which are the expected environmental concentrations after distribution in the aquatic environment. The FDA strategies are slightly different, and are based on the so called environmental introduction concentrations (EICs), which describe the concentration directly at the effluent site. EIC is usually a factor of 10 higher than the PEC. No toxicity assessment is required at this initial stage, and only if the PEC or EIC is above 0.01 µg/L or 0.1 µg/L respectively, a true risk assessment is required.

In tier 1 of the risk assessment (called phase II in the EU guideline) the fate and toxicity of pharmaceuticals are determined, and the PEC/EIC estimates may be refined. The aim is to obtain a risk quotient based on a refined PEC/EIC and a predicted no effect concentration (PNEC). First a screening is conducted, in which the toxicity of the pharmaceutical in question is determined towards the “base set” of

organisms (algae, daphnids, fish, representing primary producers and primary and secondary consumers), together with further evaluations of the physiochemical properties and fate. If antibiotics are considered, the initial risk quotient is determined separately for microorganisms (in order to protect the biological treatment step in STPs) and for the base set of aquatic organisms. A risk quotient is >1, will require further refined PEC and PNEC values in the subsequent tier. This is a case-by-case assessment that is specially tailored towards the pharmaceutical that is assessed. It should be pointed out here, that the EU guideline (in contrast to the US approaches) specifically only requests chronic toxicity data for the hazard assessment of pharmaceuticals.

When it comes to assessing the risk of antimicrobial substances, the EU guideline recommends the use of cyanobacteria (prokaryotes). However, irrespective of the intended use and mode of action of the pharmaceutical in question, the FDA guideline always starts by assessing the antimicrobial properties of the drug to ensure the proper functioning of the STP process. Antimicrobial substances are specifically mentioned in the guideline, but only as "...information regarding the toxicity to the target organism(s) should be included".

3.2 Pharmaceuticals for veterinary use

Regulatory practices of pharmaceuticals used in veterinary medicine are harmonized between the European Union, the US and Japan in the VICH cooperation (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products). The ERA is in phase I divided into an aquatic and a terrestrial branch during an initial pre-screening of environmental fate, to be further divided into three branches in the phase II testing: the aquaculture branch, the intensively reared animals branch and the pasture animals branch. For all three branches effects in the aquatic compartment might be expected from pharmaceuticals directly or indirectly introduced. Therefore, the guideline on phase II assessment includes an encompassing section on aquatic effect studies.

Similar to the strategy for human pharmaceuticals, Phase I makes use of a cut-off criterion (1µg/L in the case of veterinary pharmaceuticals), below which no risk assessment is required. Entering into Phase II, a similar risk assessment approach similar to the one for human pharmaceuticals is employed. In Tier A EC50s are determined for the base set of organisms, and the Tier B assessment aims at refining the PNEC determined in Tier A by estimating NOECs in addition to the EC50s.

3.3 Limitations of the environmental risk assessment process

These procedures have been established as a compromise, with the aim of protecting the environment, while still maintaining pragmatic usability. The following critical issues should be mentioned:

- A risk assessment only commences, if the environmental concentrations are initially estimated to be above the respective cut-off. However, it still needs to be evaluated whether the actual trigger values are suitable.
- Pharmaceuticals commonly enter the environment as part of a complex mixture, e.g. in STP effluents. It has repeatedly been shown for different types of chemical mixtures, including mixtures of pharmaceuticals (Backhaus et al., 2008; Kortenkamp et al., 2009) that a combination

of substances at concentrations not provoking any effects individually might still lead to considerable mixture effects. Therefore, the focus on a substance-by-substance assessment runs the risk of severely underestimating the actual toxicity to the exposed aquatic organisms.

- The hazard assessment of antibiotics is performed for activated sewage sludge communities only. However, such bacterial community are usually comprised of a mixture of common freshwater bacterial species together with human pathogens that end up in the sewage after excretion from the human body (Arthurson, 2008; Wagner and Loy, 2002). This has been established and endures while subjected to relatively high concentrations of antibiotics, compared to amounts commonly found in the environment. Hence, it seems likely that sewage sludge bacterial communities are more tolerant towards antibiotics than bacteria in receiving waters.
- The standard assessment factors that are sued to extrapolate between levels of biological complexity as well as inter- and intra-species variabilities are not customized to the specific properties of pharmaceuticals
- The risk of resistance/tolerance development in the aquatic environment is completely neglected.

4 | Predictive mixture toxicity assessment

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4.1 Concentration Addition and Independent Action

Mixture toxicity assessments can either be retrospective or prospective, i.e. either the hazard of a current exposure situation is determined or the effect of an expected exposure is predicted. Different approaches are employed depending on the aim of the study, commonly divided into whole mixture testing or component based approaches (Backhaus et al., 2008).

To experimentally test all potentially environmentally relevant mixtures would be a task worthy of Sisyphus, therefore predictive approaches have been proposed instead. The mathematical concepts of Concentration Addition (CA) and Independent Action (IA) both predict the toxicity of a mixture based on the individual toxicities of the mixture components (Altenburger and Greco, 2009; Faust et al., 2000), describing two mutually exclusive reference situations.

CA was derived by Loewe and coworkers in the 1920s for the pharmacology of pharmaceutical mixtures (REFs), and has therefore the advantage of a biological understanding. It is based on the assumption that the comprising substances of a mixture have a similar mechanism of action, which from a strictly mechanistic point of view makes it only valid for substances with the same molecular target (Pöch, 1993) or at least an "identical site of primary action" (Calamari and Vighi, 1992). From a broader phenomenological viewpoint, a common toxic response should be enough for the mixture to be predictable by CA (Berenbaum, 1989).

CA predicts mixture effect concentrations based on the concentrations of all the comprising substances, each scaled to a common effect level. This implies that all substances in the mixture contribute to the overall mixture toxicity, even though present at concentrations below their No Effect Concentrations (NOECs) (Boedeker et al., 1993). Hence, each of the comprising mixture substances is assumed to act as if they were dilutions of one another. CA can be mathematically formulated for an n-compound mixture as:

$$\sum_{i=1}^{n} \frac{C_i}{ECx_i} = 1$$

Where c_i denotes the concentration of compound i in a mixture that is expected to provoke $x\%$ effect, and ECx_i gives the concentration at which this compound alone provokes the same $x\%$ effect. The fraction c_i/ECx_i is also termed Toxic Unit (TU), and if a mixture is accurately predicted by CA then the sum of toxic units equals 1. The toxic unit approach result in that any component of the mixture can be exchanged by another without changing the overall toxicity of the mixture, as long as the TU of the affected substance stays the same.

Independent Action on the other hand is derived from the field of probabilistic statistics, and has no immediate biological basis. The foundation of the concept is the estimation of the overall probability of a number of independent random events to happen. Translated into the field of ecotoxicology, this means that the overall effect of a mixture is determined by the probability that each of the comprising substances cause an effect. Due to its probabilistic basis, IA assumes that all substances in a mixture exert their effects completely independent of each other. This is usually interpreted as the compounds affecting different biological pathways (Bliss, 1939) or different main physiological and life-history traits (Barata et al., 2007).

When inhibition of an endpoint is determined (i.e. increasing concentrations cause increasing effects), the effect of a mixture comprised of *n* compounds is calculated by applying the statistical concept of independent random events (Bliss, 1939):

$$E(c_{\text{Mix}}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)]$$

Where $E(c_i)$ is the effect of compound *i* if applied alone at concentration c_i, the concentration at which it is present in the mixture. Hence, IA only takes substances present in the mixture at concentrations high enough to cause an effect into account if applied singly, as opposed to CA. It should be noted that IA predicts effects, whereas CA predicts effect concentrations. Therefore, the numerical outcome of the two predictions cannot be immediately compared, but one transformed into the same unit as the other. The two concepts also differ in their demand on the input data for the predictions. Since CA is based on the toxic unit approach, only a common effect concentration (e.g. the EC50) is needed for all the mixture components and then all concentrations can be related to that. IA on the other hand needs effects of the individual mixture components at all concentrations by which they are present in the mixture.

CA has been extensively investigated, and described mixture toxicity best for specifically similarly acting substances such as pesticides e.g. (Junghans et al., 2006), pharmaceuticals e.g. (Cleuvers, 2004) and endocrine disrupting chemicals e.g (Kortenkamp, 2007), as well as unspecifically acting, so-called baseline toxicants, e.g. (Hermens et al., 1984).

The empirical evidence on the performance of IA is much more limited, but effects of multi-component mixtures of dissimilarly acting chemicals on the marine bacterium *V. fischeri* (Backhaus et al., 2000a) and freshwater algae (Faust et al., 2003) were clearly better described by IA. Irrespective of which concept explains the observed mixture toxicity better the ratio between IA- and CA-predicted EC50-values is usually not more than a factor of 5 (Kortenkamp et al., 2009), and in studies with binary combinations IA and CA predicted nearly indistinguishable mixture toxicities in many cases (Cedergreen et al., 2008).

In all the studies mentioned above, when both concepts have been used for predicting toxicities, CA is the most conservative concept predicting higher toxicity of the mixture than IA. That, together with

the lower data demand of CA, has lead to the proposal of CA as a default concept to use in environmental risk assessment of mixtures (Backhaus et al., 2008).

4.2 Interactions

A prerequisite for the predictive concepts to be valid is that the mixture components do not interact, i.e. their toxicities cannot be affected by the other mixture substances.

However, pharmaceuticals are known to interact in several ways: by chemical interactions between the compounds, toxicokinetic interactions (i.e.interactions in uptake, metabolism and excretion of the substance and toxicodynamic interactions (i.e. interactions at the target site). The consequence of such interactions can either be an increase in toxicity (usually referred to as synergism) or a decrease (antagonism). Hence, interactions are usually classified as synergistic or antagonistic, but a claim on synergy or antagonism can only be valid in relation to an expected outcome. Therefore when predicting mixture toxicities, synergy is only a correct description when the toxicity is higher than predicted by both concepts, and vice versa for antagonism. Synergy in a community context can sometimes also denote an increased spectrum of activity, i.e. species insensitive to the comprising substances when applied singly becomes affected by the mixture. A special case of synergy is so-called potentiation, when a non-toxic substance combined with a toxic substance produce a higher toxicity than the toxic substance alone (Chou, 2006).

When trying to establish what predictive concept is the most accurate one for describing mixture toxicity there is always a dual interpretation of the outcome, the so-called assessment dilemma. For example, if the toxicity of a mixture is accurately predicted by CA, this does not necessarily mean that the mixture substances have similar mechanisms of action since there can be interactions between the mixture components, driving the toxicity away from the IA prediction.

From clinical research, binary combinations of antibiotics are known to interact in both synergistic and antagonistic ways (Yeh et al., 2006). In fact, e.g. sulfamethoxazol and trimethoprim are actually administered together due to their proposed synergistic properties.

For multi component mixtures like the ones generally encountered in an environmental setting, no data is published on interactions. A few ecotoxicologocal studies have been published on synergistic or antagonistic effects when green microalgae were exposed to binary combinations of antibiotics. Synergy between sulfa drugs and trimethoprim was shown by Eguchi and colleagues (Eguchi et al., 2004), and antagonistic effects were seen in combinations of oxytetracycline and flumequine and flumequine and erythromycin (Munch Christensen et al., 2006).

Substances with potentiating properties are within clinical science called chemosensitizers, i.e. substances not toxic themselves but with the ability to increase the effect of pharmaceuticals. These were deemed deserving "…a top rank among environmentally-hazardous chemicals…" when discovered in 1995 by Kurelec and collegues (Kurelec et al., 1995). The basis of the action of chemosensitizers is the fact that all living organisms share a basic defense system that helps them survive in contaminated environments, the so-called multixenobiotic resistance (MXR) (Epel et al., 2008). This is based on ef-

flux pump systems situated in the cell membrane that act as bilge pumps, extruding potentially harmful substances from the cell. One well known example is the multidrug resistance (MDR) proteins responsible for anti-cancer drug resistance in tumor cells, a major obstacle in successful treatment of cancer. Also the resistance against antibiotics that has emerged in infectious bacteria is in many cases based on efflux (Nikaido and Takatsuka, 2009). To combat these, chemosensitizers have been developed that interact with the pump systems and prevents extrusion. Due to their potential application within human medicine they are designed not to be toxic themselves, only to potentiate the effects of other drugs.

However, the phenomenon of chemosensitization is not only restricted to a controlled clinical setting but has also emerged (as previously mentioned) as a potential environmental problem. While inhibition of efflux is beneficial in cancer therapy, it might be detrimental for organisms living in contaminated environments. For many aquatic organisms MXR is crucial for coping with stress from e.g. pesticides, metals and organic substances (Kurelec e.g.). If there also are substances present that act as chemosensitizers, the effect of the stressor may be larger than anticipated. Synthetic musk substances are ubiquitous in the aquatic environment due their extensive use in cosmetics and personal care products, but generally regarded as low risk in environmental risk assessment (Heberer, 2003). However, a few years ago Luckenbach and colleagues reported that synthetic musk substances inhibited MXR in mussels (Luckenbach et al., 2004). Since then, chemosensitizing properties of both naturally occurring substances (Timofeyev et al., 2007) and contaminants (Epel et al., 2008) have been reported for eukaryote aquatic organisms.

Whether chemosensitization occurs also in bacteria in the aquatic environment is so far unknown, but as previously mentioned all bacteria have genes coding for general efflux mechanisms in their chromosomal DNA. These belong to five families: the resistance-nodulation-division proteins (RND), the major facilitator superfamily (MFS), ATP binding cassette family (ABC), multidrug and toxic compound exporters (MATE) and the small multidrug resistance (SMR) family. Of these, RND transporters play the greatest role for antibiotic resistance in gram negative bacteria, whereas MFS transporters are the most important pumps in Gram positive bacteria (but present in both) (Marquez, 2005; Nikaido, 2009).

No chemosensitizers have been approved for clinical use in antibiotic treatments, but several pharmaceuticals have been shown to inhibit antibiotic efflux in bacteria e.g. phenothiazine drugs, paroxetine and Verapamil (Couto et al., 2008; Pages and Amaral, 2009; Sabatini et al., 2008). The increase of toxicity caused by the efflux inhibiting substances has in some cases been shown to be substantial, e.g. the combination of the phenothiazine drug chlorpromazine with the antibiotic oleandomycin decreased the MIC (minimum inhibitory concentration) 8,000 times (Chan et al., 2007). However, all bacterial studies assessing chemosensitization are performed using highly resistant pathogens together with concentrations of both antibiotics and inhibiting substances vastly above any environmentally relevant. The pharmaceuticals shown to inhibit bacterial efflux are all detected in STP effluents (REFs). Hence, the co-existence of antibiotics and chemosensitizers might be of environmental concern, not only in terms of enhanced toxicity of the antibiotics towards bacteria in the aquatic environment, but it would also challenge the use of predictive mixture toxicity assessment of antibiotics.

5 | Aims and approaches

The overall aim of this thesis was to assess the potential risk of pharmaceuticals and pharmaceutical mixtures for natural microbial communities in the aquatic environment. The main focus was on the significance of the limitations of current risk assessment strategies when it comes to effects of antibiotics and sensitivities of natural microbial communities.

The following main questions were addressed:

- Are current levels of pharmaceuticals present in the aquatic environment a risk for natural microbial communities?
- Is there a risk of bacterial tolerance/resistance development caused by antibiotics contaminating the aquatic environment?
- Are current risk assessment strategies sufficient to protect environmental microbes?
- Is the predictive power of the concepts of CA and IA sufficient for accurately predict effects of pharmaceutical mixtures on natural microbial communities?
- Are interactions to be expected in pharmaceutical combinations?

In order to assess the risk of current levels of pharmaceuticals, effects on both specific and more integral endpoints need to be assessed. Therefore, both short-term toxicity of protein biosynthesis antibiotics and chronic toxicity of five pharmaceuticals and personal care products were assessed. Bacterial resistance development is an unusual endpoint, but has a high ecological relevance when it comes to antibiotics. Therefore, natural microbial communities were exposed to relatively high concentrations of antibiotics in order to assess the potential for tolerance development in addition to any indications of resistance development of the bacterial species in the community.

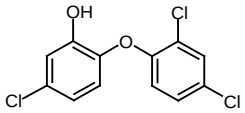
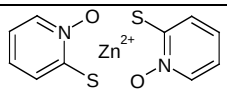
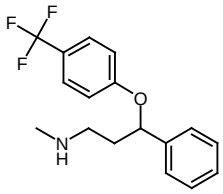
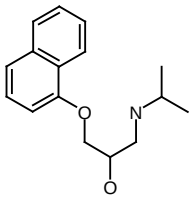
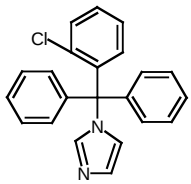
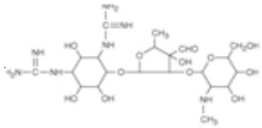
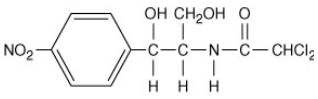
The importance of including mixtures in environmental risk assessment has started to become widely acknowledged, but in order to use predictive approaches they must be shown to be valid for common exposure situations such as microbial communities exposed to pharmaceutical mixtures. Therefore, also the predictability by CA and IA of the mixture toxicity was assessed in the short-term and the chronic study. In addition, increased toxicity of antibiotics to bacteria have been shown when co-exposed to so-called chemosensitizing substances in a clinical setting, therefore this potential was assessed also for natural microbial communities. In short, the papers addressed these issues as follows:

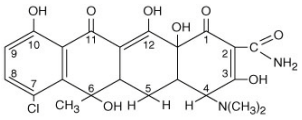
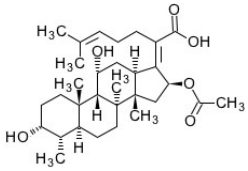
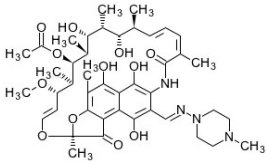
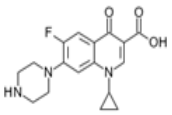
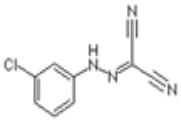
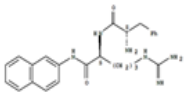
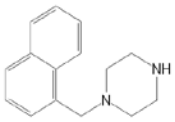
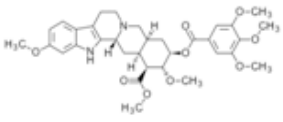
1. Hazard assessment of selected pharmaceuticals
    a. Short-term toxicity (paper I) (acute toxicity usually is lethality/mortality)
    b. Chronic toxicity (paper II, III, IV)
    c. Tolerance and resistance development towards antibiotics (paper III)
2. Evaluation of mixture effects of pharmaceuticals (paper I, II)
    a. Short-term toxicity (paper I)
    b. Chronic toxicity (paper II and III)
3. Predictability of mixture toxicity (paper I, II)
4. Potential for synergistic interactions (paper IV)

6 | Methodological considerations

6.1 Test substances

Table 1. Details on chemical structure and mechanisms of action for the pharmaceuticals and chemo-sensitizers used in the studies of this thesis are shown

Name	MW (CAS)	Structure	Mode or mechanism of action
Triclosan	289.5 g/mol (380-34-5)		inhibition of lipid biosynthesis by blocking the enoyl-acyl carrier protein reductase (ENR)
Zinc-pyrithione	317.7 g/mol (13463-41-7)		membrane depolarization
Fluoxetine	345.8 g/mol (59333-67-4)		selective serotonin re-uptake inhibitor in mammals
Propranolol	257.3 g/mol (525-66-6)		non-selective ß-blocker in mammals
Clotrimazole	344.8 g/mol (23593-75-1)		inhibitor of cytochrome P450 dependent 14-demethylase in fungi
Streptomycin	7307 g/mol (5490-27-7)		Antibiotic. Impairs amino acid specificity In protein synthesis
Chloramphenicol	323.1 g/mol (56-75-7)		Antibiotic, Inhibits peptide bond formation in protein synthesis

Chlorotetracyc-line-hydrochloride	515.3 g/mol (64-72-2)		Prevents the tRNA from attaching to the ribosome
Fusidic acid	538.7 g/mol (751-94-0)		Prevents translocation of EF-G from the ribosome
Rifampicin	823.0 g/mol (13292-46-1)		Blocking elongation of RNA chain
Ciprofloxacin	331.3 g/mol (85721-33-1)		Fluoroquinolone antibiotic, Inhibits DNA replication
CCCP	204.6 g/mol (555-60-2)		Uncouples membrane proton gradient
PAβN	519.5 g/mol (100929-99-5)		Inhibits RND pump systems
NMP	226.321 (40675-81-8)		Inhibits RND pump systems
Reserpine	608.7 g/mol (50-55-5)		Inhibits MFS pump systems

In the study of paper I, five protein biosynthesis inhibiting antibiotics (streptomycin, chloramphenicol, chlortetracycline, rifampicin and fusidic acid) were chosen to assess the predictability of acute toxicity of antibiotics on planktonic bacterial communities. They all share general mode of action (inhibition of protein biosynthesis) but have distinctly different molecular targets.

In paper II, when assessing chronic toxicity of pharmaceuticals and personal-care products (PPCP) on algae in periphyton communities, five substances of common use previously shown to be toxic to green algae were chosen: fluoxetine (anti-depressant, pharmaceutical) , propranolol (lowering blood-pressure, pharmaceutical), triclosan (broad spectrum biocide, used to prevent microbial growth in e.g. XXX), zinc-pyrithione (broad spectrum biocide, used e.g. in anti-dandruff shampoo) and clotrimazole (anti-fungal drug, pharmaceutical). For all but propranolol and fluoxetine, the mechanism of action in algae is known, and all of them are expected to be distinctly dissimilarly acting.

The main toxicant in paper III and IV was ciprofloxacin, a fluoroquinolone antibiotic inhibiting DNA replication in bacteria. It is also commonly found in sewage effluents (se Tab XX), is a known inducer of bacterial resistance (Jacoby, 2005) and also a known substrate for bacterial efflux (Alekshun and Levy, 2007).

The effluent from the Patancheru STP tested in paper III contains high concentrations of pharmaceuticals, where ciprofloxacin is present at highest concentrations (around 100 µmol/L), followed by losartan at 6 µmol/L and Cetirizine at 4 µmol/L. Of the remaining 8 of the top 11 pharmaceuticals detected, 5 are fluoroquinolones at concentrations between 2.5 µmol/L and 0.5 µmol/L.

The chemosensitizers used in paper IV, Phe-arg β-naphthylamide dihydrochloride (PAβN or MC207,110), 1-(1-naphthylmethyl)piperazine (NMP) and reserpine, were selected based on their target pump systems. The first two have been shown to inhibit efflux of RND pump systems (REF), whereas reserpine inhibits pumps of the MFS family (REF).

6.2 Strategies for determining effects on bacteria and algae

In paper I, plankton communities were sampled simply by grab samples from a lake. In a lake, there are no physical boundaries except for the lake floor and surface, and microbial ecologist therefore often treat all organisms in the in the water column as part of one community although not necessarily interacting. Still, when grabbing a water sample a "new" community will be defined by the organisms present. The antibiotics tested all inhibit protein biosynthesis in bacteria, a specific mode of action possible to directly measure by the radiolabelled L-leucine incorporation technique commonly used to assess activity of bacterial communities in microbial ecology. Hence, acute effects (2h) of the antibiotics could be measured as differences in incorporated leucine in the total protein content of the bacterial community between the exposed and control communities.

In the other papers, marine (paper II) or limnic (papers III and IV) periphyton communities were sampled on small circular glass discs submerged into the water for around 7 days, brought to the lab and exposed to toxicants for 72h (limnic periphyton) or 96h (marine periphyton) in the semi-static SWIFT test developed by (Porsbring et al., 2007). This is designed to mimic the toxicant induced suc-

cession of communities in the environment, but in a shorter time-frame. The changes in community structure after SWIFT would preferably be measured by direct species count. However, for the algae this is extremely labour intensive and requires highly specialized taxonomic skills. Therefore, the changes in algal and cyanobacterial species composition were approximated by analyzing the pigment content of the community, a method providing relatively high throughput, proven to be a good separator of algal classes, groups of species or processes occurring in phytoplankton populations (Wright et al., 1991). Effects and effect concentrations needed as input for the predictions were then assessed for the content of each pigment in relation to the content of the same pigment in the control treatments.

A bacterial species is defined by its genetic similarity to other bacteria, and hence impossible to visually determine without fluorescence labelling or colouring techniques. Therefore, in order to assess changes in the prokaryote part of the periphyton communities, the carbon utilization pattern of the community was determined. This cost-efficient compromise between function and structure has been developed in the Biolog Ecoplate™ system (Biolog Inc, from here on referred to as Ecoplates), where a 96-well microtiterplate has been coated with 3*31 different carbon substrates together with a dye that turns purple upon oxidation. The assumption is that different groups of bacteria will be able to utilize different carbon substrates, and hence the wells with degraded substrates will gradually turn purple. Even though developed for soil microbial communities, the method has been shown to distinguish between different kinds of communities also in freshwater environments (REFs). Even though wrought with confounding factors when used to assess microbial diversity between different sites, it is well suited for the control-treatment comparison in the studies of this thesis (Preston-Mafham et al., 2002).

The Ecoplates provide a time series of colour development of the different wells of each plate. This can either be collapsed into a general response of the community, the average well colour development (AWC), or the response of each carbon source can be evaluated. In the thesis both strategies were employed, the latter by fitting a curve to the colour development over time based on all replicates for each well and treatment. The area under the colour development curve (AUC) was determined by integration. For each treatment the inhibition of colour development was determined. In paper III the concentration-response relationship for each toxicant was determined by curve fitting, and finally EC50s determined. The resulting values were used as input for a Principle Component Analysis (PCA), which transforms the possible correlated multivariate input data into a number of uncorrelated principle components that each explains a certain amount of the variability of the data. The outcome is a set of coordinates that project the different treatments into a coordinate system, making it easy to qualitatively distinguish between the outcomes of the different treatments. In paper IV, the inhibition of AUC was used as input for the IA predictions.

6.3 Tolerance development

In paper III, the effects of the effluent from the Patancheru STP on limnic periphyton were assessed in parallel to ciprofloxacin alone at corresponding concentrations. Sensitivity of the communities was assessed by exposing them to a range of concentrations of either toxicant in the Ecoplates. The tolerance induction of either the effluent or ciprofloxacin was investigated by first exposing the communities to a fixed concentration of either ciprofloxacin or the effluent in SWIFT, followed by exposure to a

range of concentrations of either the effluent or ciprofloxacin in the Ecoplates. Seven different exposure combinations were evaluated in order to compare what different pre-exposures would do to the bacterial communities.

6.4 Mixtures

The mixture studies in the thesis were mainly focussed on predictive assessment, i.e. except for in paper III no evaluation of present exposure situations was made. For the predictive studies a component based approach was employed where artificial mixtures have been created in the lab in order to control which, and at what concentrations, the substances comprising the mixture are present at. In paper I and II a so-called fixed ratio design of the mixture was applied, where the relative amount of the substances is always the same but the total mixture concentration varied to cover the whole effect range (Altenburger et al., 2000; Backhaus et al., 2000a). This method is commonly applied for multi-component mixtures with the aim to validate the predictive approach for a certain mixture or test system. It does however fail to detect any concentration-ratio dependent deviations from the predictions. The other option would be to assess toxicity over the whole response surface, i.e. vary both ratios between the mixture substances and the total mixture concentration (Jonker et al., 2005). This approach is most common for studies of binary combinations because of the rapidly increasing number of samples needed when increasing the number of mixture substances. In addition, the visualization of a concentration-response surface becomes a challenge when the number of mixture substances increases, e.g. the five component mixture tested in paper I would require a plot with 6 dimensions.

The molar ratios selected for the mixtures in paper I and II were based on the EC50s and NOECs of the comprising substances. Since both were designed with the intent of assessing predictability of the mixture toxicity, the aim was to obtain an equal contribution of all the mixture substances to the overall mixture toxicity, not to assess a realistic environmental exposure situation.

A different approach was taken when assessing the risk of the effluent released from the STP coupled to pharmaceutical production in the Patancheru region in India (paper III). A retrospective toxicity assessment was made by exposing periphyton communities to dilutions of the whole effluent. The effects were then compared to the toxicity of the main component of the effluent, the antibiotic ciprofloxacin.

6.5 Chemosensitization

Antibiotics are substances prone to interact. Since synergy is a sought after phenomenon in clinical studies, this has been extensively investigated. However, the definition of synergy is somewhat arbitrary for some methods and the reasons behind the synergy in many cases unknown. However, when it comes to chemosensitization, both the mechanisms and the outcome of the studies are clearly defined. Therefore, three substances clearly inhibiting efflux of the antibiotic ciprofloxacin in clinical studies were selected to assess the impact of chemosensitization on the combined toxicity.

7 | Main results and discussion

Table 2. A summary of the toxicity of the pharmaceuticals tested, compared to measured concentrations in waste waters and effluents. N.d. denotes data not determined or not available.

				Detected (nmol/L)		MEC/NOEC	
Substance	**Endpoint**	**NOEC nM**	**EC50 nM**	**MECMedian**	**MEC95%ile/ MECmax**	**median**	**max**
Paper I							
Chloramphenicol	Leucine incorporation	74	5698 [5050-6370]	3.126	7.520	0.042	0.1016
Chloramphenicol	Leucine incorporation	212	3388 [2550-3820]	3.126	7.520	0.015	0.0355
Chlorotetracycline	Leucine incorporation	< 10	138.0 [114.0-0.200]	168.0	8979	16.80	897.9
Chlorotetracycline	Leucine incorporation	10	496.0 [303.0-577.0]	168.0	8979	16.80	897.93
Fusidic Acid	Leucine incorporation	10	4550 [2820-6760]	n.d.	n.d.	n.d.	n.d.
Fusidic Acid	Leucine incorporation	1	1041 [872.0-1191]	n.d.	n.d.	n.d.	n.d.
Rifampicin	Leucine incorporation	28	359.0 [80.00-390.0]	n.d.	n.d.	n.d.	n.d.
Rifampicin	Leucine incorporation	<3	959.0 [670.0-1131]	n.d.	n.d.	n.d.	n.d.
Streptomycin	Leucine incorporation	3170	47900 [35800-64900]	n.d.	n.d.	n.d.	n.d.
Streptomycin	Leucine incorporation	9950	79100 [37100-150000]	n.d.	n.d.	n.d.	n.d.
Paper II							
clotrimazole	tot. pig. cont.	10	441.2 (350.9 - 535.9)	0.0667	0.0960	0.007	0.0096
triclosan	tot. pig. cont.	10	1166 (850.7 – 1481)	0.1761	n.d.	0.018	n.d.
Zn-pyrithione	tot. pig. cont.	1	7.245 (6.547 - 7.876)	n.d.	105	n.d.	105
fluoxetine	tot. pig. cont.	20	111.6 (93.30 - 130.9)	0.0272	0.3201	0.001	0.016
propranolol	tot. pig. cont.	100	323.8 (289.4 - 353.6)	1.068	25.06	0.011	0.2506
Paper III							
Ciprofloxacin	Ecolog	1.5	47.3	0.7575	107.5	0.505	71.69

7.1 Sensitivities of the test systems

When possible, the EC50 and NOEC values determined in the papers of this thesis were compared to previously published studies, to assess the sensitivity of the different assays used. In general, the effect concentrations of the tested pharmaceuticals were at equal or lower concentrations then previously determined. However, most likely due to inherent insensitivities of the more abundant species of the communities, especially streptomycin and triclosan were less toxic to the communities tested than in previous studies (see data and references in paper I, II and III).

7.2 Environmental risk of individual pharmaceuticals

A rough estimation of environmental risk of the tested substances can be made by comparing measured environmental concentrations (MECs) with the NOECs determined in this thesis. Lindberg et al followed this procedure using concentrations detected in STPs across Sweden (Lindberg et al., 2007). This approach corresponds to the risk quotient approach used in regulatory risk assessment, where a ratio above 1 indicates environmental risk. Table 2 summarize the MEC/NOEC ratios where data on environmentally detected concentrations were available. Two ratios are given, first the MEC_{median} which is based on the median concentration detected in the included studies. For the ciprofloxacin, the maximum concentrations detected were several orders of magnitude from the 95%ile (i.e. the concentration below which 95% of the observations fall), therefore the $MEC_{95\%ile}$ was used for all the antibiotics, instead of the MEC_{max} as for the other pharmaceuticals. In addition, it should be noted that the concentrations in ref (Segura et al., 2009) includes incoming raw sewage, and the treatment process might remove a certain amount. However, depending on the treatment process, this amount varies extremely much for the investigated substances. Reviews by Miege and Onesios together with a long term study by Gros showed that removal of ciprofloxacin varied between 37-99%, tetracycline between 35-89% removal of chloramphenicol between 45-93% (Gros et al., 2010; Miege et al., 2009; Onesios et al., 2009). Therefore, an assessment can still be made using these detected data if taking removal into consideration.

As can be seen in Tab 2, the only substance which poses a clear risk towards bacterial communities in the aquatic environment is chlortetracycline, with $MEC_{95\%ile}$/NOEC and MEC_{median}/NOEC ratios of 898 and 16.8 respectively, and even a MEC_{median}/EC50 of 1.2. Even considering the average removal of chlortetracycline (60% reduction) the median concentration would be 67 nmol/L, a concentration that would cause almost 50% effect according to the results in paper I. In addition, the 95%ile concentration shows that fluctuations in chlortetracycline concentrations can be extreme, and while reducing $MEC_{95\%ile}$ by 60%, the concentration is still 3592 nmol/L resulting in a MEC/NOEC ratio of 359.

Also zinc pyrithione seems to be indicated as of high environmental risk towards natural microbial communities, however not because of its use as a personal-care product but as active substance in antifouling paints. Since it is extremely hard to analyse due to technical difficulties using common analytical methods, only one published study sampling water from a marina situated within a dock provides environmental concentrations (Mackie et al., 2004; Thomas and Brooks, 2010). This concentration would result in a MEC/NOEC ratio of 131 (and result in a complete reduction of total pigment content according to paper II), but cannot be regarded as the common exposure situation because of the sampling site.

At the other end of the scale are the substances where the MEC_{max}/NOEC ratio indicate a low environmental risk for natural microbial communities even at high concentration events, e.g. clotrimazole (0.003), chloramphenicol (0.04-0.1) and fluoxetine (0.4).

For both propranolol and ciprofloxacin, the assessment turns out differently depending on which detection data is used. For propranolol MEC_{max} /NOEC equals 31, whereas MEC_{median}/NOEC produce a ratio of 0.01. For ciprofloxacin NOEC were not estimated, but the EC01 (taken as a rather conservative surrogate for the NOEC) is 1.5 nmol/L. The $MEC_{95\%ile}$/EC01 and MEC_{median}/EC01 then becomes 71.9 and 0.505 respectively. Hence, environmental risk of these substances can be expected for natural microbial communities at presently occurring high concentration events.

In general, more information is available on environmental concentrations of the antibiotics than the other pharmaceuticals. Therefore, that the NOECs determined is above environmentally detected concentrations does not necessarily mean that they are of low environmental concern, but that the detection data is too limited.

The integral responses measured (algal biomass, average bacterial community response) was also analysed on a more detailed level. Then, it becomes obvious that more specific endpoints also are more sensitive. When measuring sterol content of the algal community exposed to clotrimazole (i.e. when directly measuring the at the action site) there were strong effects already at 0.5 nmol/L. For ciprofloxacin, EC50 of two carbon sources was at concentrations <3 nmol/L. Hence, an evaluation of environmental risk is very much endpoint dependent.

In addition, risk is also very much exposure scenario dependent. In the “normal case” i.e. at median concentrations of the tested substances only chlortetracycline has a MEC/NOEC above 1. However, the 95%ile and maximum concentrations indicate that there are presently occurring events of much higher concentrations, which constitute a definite risk towards the microbial communities also for ciprofloxacin, zinc pyrithione and propranolol.

7.3 Effects of the effluent from bulk drug production

The effluent proved to be severely toxic to the periphyton, where the ciprofloxacin content at $EC50_{AWC}$ of the effluent (47 nmol/L) is in the region of ciprofloxacin concentrations detected about 30 kilometers downstream the sewage treatment facility in the Nakkavagu River (30 nmol/L). Samples from a well used as drinking-water supply in the same area contained 3.3 nmol/L ciprofloxacin (Fick et al., 2009), which is close to $EC10_{AWC}$ in the present study.

Based on the average response of the community (inhibition of AWC), ciprofloxacin alone seemed to be the driving force of the effluent toxicity. However, analysis of the response of the individual carbon sources showed that ciprofloxacin and the effluent in fact induce different responses of the exposed communities. The highest tolerance development was seen towards ciprofloxacin by communities pre-exposed to the effluent in SWIFT. The ability of the effluent to induce tolerance towards itself was lower. When only taking the AWC into account, the tolerance development towards ciprofloxacin by the ciprofloxacin pre-exposure seemed negligible. However, the more detailed analysis showed the

interesting pattern that actually induced tolerance in a part of the community to a high extent whereas the effluent induced lower tolerance but in a larger part of the community. Whether the induced tolerance is due to resistance development of the bacterial species comprising the community exposed cannot be determined for certain only by the Ecoplates, but since at least some species selectivity of the Ecoplates has been suggested (Christian and Lind, Garland 1997) the results may indicate this. However, further studies are needed to bring clarity into the matter.

7.4 Effects and predictability of pharmaceutical mixtures

In paper I, the predictability of acute effects on bacterioplankton communities of a mixture of antibiotics was assessed. The specific aim was to investigate the outcome of a mixture comprised of substances with the same mode of action, but distinctly different target sites. CA was the better predictor of mixture toxicity, which would be expected taking the viewpoint that a common toxic response is enough for CA to be valid. However, the result could also be interpreted as antagonistic effects shifting the predictions away from IA (the so-called assessment dilemma). In any case, neither concept was off by more than a factor of 1.5 at EC50 of the mixture. Unusually enough, IA predicted a higher toxicity than CA due to the flat slopes of the single substance concentration response curves.

Paper II assessed predictability of the chronic toxicity of a mixture of PPCPs on periphyton communities. The substances were assumed to have dissimilar modes of action, even though the molecular target in algae is unknown for two of them (propranolol and fluoxetine). Therefore, it was the first time a study investigated the combined effects of dissimilarly acting pharmaceuticals on a community level of biological complexity. At the mixture EC50 both IA and CA predicted almost equal toxicities, but at lower effects a comparison proved to be problematic due to prominent hormesis effects of the mixture. Also when assessing the effects of a mixture of distinctly dissimilarly acting substances on carbon fixation by epipsammon communities, hormesis was seen (Backhaus et al., 2004). Hence, it seems to be a recurring phenomenon in community studies. When occurring also in the concentration response curves of the single substances, it obstructs the ability of IA to predict mixture effects (i.e. it violates the probabilistic basis of the concept, since there are no negative probabilities). Hormesis is seen within a wide range of biological disciplines and is generally acknowledged as an adaptive stress response within the individual, which at chronic exposures in many cases lead to a decreased fitness (Calabrese, 2008) and hence a high biological cost. In community ecotoxicology, hormesis like effects may also be due to indirect effects, e.g. in paper II where increase in algal biomass is suspected to be due to top-down effects from reduced grazing (i.e. fluoxetine and propranolol is toxic to the grazers). Hence, the dynamics of the whole community seems to be shifted.

When the PPCPs were combined at their individual NOECs, a mixture effect of 28% on total algal pigment content was observed. Although previously observed for similarly acting pharmaceuticals, e.g. 10 fluoroquinolones combined at their individual NOEC caused 99% effect (Backhaus et al., 2000b), this has not been shown previously for dissimilarly acting pharmaceuticals. Whereas only five substances were combined in paper II, the common exposure situation an STP effluent is at least to 15-20 pharmaceuticals simultaneously (Andreozzi et al., 2003). If each of them were assigned a generic NOEC based on the average NOEC of the substances in paper II (approximately 6%), the expected mixture toxicity according to IA would be ranging between 60-70% for the 15-20 substances.

Paper IV assessed the impact of chemosesitizing substances on ciprofloxacin uptake and toxicity in periphyton communities. Based on the results from clinical studies, where the Minimum Inhibitory Concentration (MIC, i.e. the lowest concentration reducing all growth) for fluoroquinolones were reduced between by a factor of four and a factor of eight (Kern et al, 2006; Schmitz et al, 1998; Lomovskaya et al 2001), the expected outcome was that the combination of ciprofloxacin and chemosensitizers should increase the uptake, and increase both acute and chronic toxicity. The observed outcome turned out somewhat differently. First of all, as opposed to the clinical studies, the chemosensitizers were highly toxic towards the environmental bacteria at concentrations commonly used. Therefore, no simple comparison of the different effects caused by pure ciprofloxacin and the combination ciprofloxacin-chemosensitizer could be made. Instead, due to the likelihood of different toxic mechanisms of action, the expected combined toxicity was predicted by Independent Action. In the uptake studies, the combination caused a lower uptake of ciprofloxacin than the controls. In both acute and chronic assays, no toxicity beyond the predicted could be seen. Rather, when assessing the response of the individual carbon substrates a significantly lower toxicity than predicted was seen for two of the treatments. Hence, chemosensitization seems to be of low relevance when it comes to ecotoxicity of ciprofloxacin towards natural bacterial communities. In addition, the study does not provide any reason not to use predictive approaches when assessing toxicity of combinations with ciprofloxacin.

8 | Conclusions

The overall aim of this thesis was to assess the potential hazard of pharmaceuticals and pharmaceutical mixtures for natural microbial communities in the aquatic environment. To conclude, I will therefore go through the main questions addressed.

8.1 Are current levels of pharmaceuticals present in the aquatic environment a risk for natural microbial communities?

Since the term pharmaceutical encompasses such a great expanse of different chemical groups, no general conclusions on "pharmaceuticals" can be made. It is clear from these studies that inference of hazard is very much endpoint and scenario dependent, and that detection data for certain classes of pharmaceuticals are still lacking.

Of the tested substances there are strong indications on environmental hazard for microalgae at high concentration occurrences of zincpyrithion and propranolol. For triclosan, fluoxetine and clotrimazole hazard cannot be excluded due to the general lack of environmental detection data for these substances. For the antibiotics tested, chlortetracycline causes high effects already at median concentrations currently detected, and should hence be considered hazardous towards environmental bacterial communities. The higher concentrations detected of ciprofloxacin caused severe effects on the bacterial communities in this thesis, whereas median concentrations were below NOEC. Hence, hazard of chronic exposure of ciprofloxacin cannot be excluded.

In addition to the hazard from exposure towards individual substances, the results from NOEC mixture of the five PPCPs highlight the importance of taking combined effects into account. Hence, environmental hazard of pharmaceuticals cannot be excluded even if only present at low (pico-nmol/L) concentrations.

The effects of the Patancheru STP effluent on the other hand are severe even at high dilutions (EC50 at 0.06%) . It is clear that the extreme amounts of antibiotics released from the bulk drug production in the region must cause enormous effects on the bacterial community exposed, not only at the release site but also in the whole region.

8.2 Is there a risk of bacterial tolerance/resistance development caused by antibiotics in the aquatic environment?

When exposed to high (100 nmol/L resp 0.107% of sterile filtered effluent from the drug production facilities in Patancheru, India) concentrations of antibiotics, there was a clear tolerance development in the bacterial communities, possibly due to resistance development of some species in the community. Since the effluent caused higher tolerance towards ciprofloxacin than ciprofloxacin itself, the exposure situation outside the Patancheru TP is especially likely to induce tolerance in the exposed communi-

ties. It might also imply that a combined exposure of antibiotics might induce higher tolerance to specific antibiotics than exposure to that antibiotic alone. However, little is known about tolerance development at the combined exposure to low concentrations of antibiotics, i.e. the common exposure situation. However, sub-inhibitory concentrations are known to cause responses on e.g. cell functions and change the genetic expression of virulence factors or the transfer of antibiotic resistance (Ohlsen et al., 1998; Salyers, 2002), but what this implies for resistance development in natural microbial communities needs to be further investigated.

Currently, the discussion mainly regards whether resistance may develop in sewage treatment plants since that is where bacterial communities encounters the highest concentrations of antibiotics (Kummerer, 2009b). Before this can be judged in either direction, studies including natural bacterial communities exposed to common environmental concentrations must be performed where molecular studies are made to pinpoint the mechanisms behind the responses.

8.3 Are current risk assessment strategies sufficient to protect environmental microbes?

Environmental risk assessment rarely deals with effects on individuals, but has the ultimate goal to protect species diversity and ecosystem function. From that perspective, at least three major weak spots of current regulations can be pinpointed based on the results in this thesis.

First of all, there is currently a difference in the aim of regulatory risk assessment of pharmaceuticals in general and antibiotics specifically. Where the first is to protect the environment, the second is only performed with the intention of ensuring function of the biological degradation in STPs. In this thesis, all results points towards the same conclusion: natural microbial communities from pristine environments are generally more sensitive towards the pharmaceuticals tested than other test systems, especially compared to the sewage sludge assay when available for comparison.

Secondly, the extrapolation approach used today is challenged by the extremely flat concentration response curves of antibiotic effects of bacterial communities. Even an assessment factor of 1000 from EC50 of fusidic acid (paper I) would not suffice to reach a concentration not causing any effects. Even when the PNEC estimation is based on a NOEC, the common assessment factor of 10 might not be enough, since the NOEC has no biological significance but depends completely on the experimental setup, see e.g. (Crane and Newman, 2000).

Thirdly, no mixture effects are taken into account. The 5 PPCPs mixed at the NOEC of each substance provoked a significant effect of 28%, (once again) pointing to the fact that mixtures matter.

Hence, neither the current regulations on environmental risk assessment of pharmaceuticals in the European Union nor the US are enough to protect natural microbial communities. However, the European guidelines are more conservative by requiring NOEC values of chronic studies, thus accounting for the specific mechanisms of action of pharmaceuticals.

8.4 Is the predictive power of the concepts of CA and IA sufficient for accurately predicting effects of pharmaceutical mixtures on natural microbial communities?

Both short-term effects of the antibiotic mixture and chronic toxicity of the 5 PPCPs were well predicted by the concepts. For the former, CA was the better choice (even though IA only underestimated the toxicity by a factor of 1.5 at the mixture EC50). IA and CA predicted almost identical mixture toxicities at the mixture EC50 of the PPCP mixture, even though effects were generally better predicted by IA over the whole effect range. Thus, the concepts seem to be applicable also for pharmaceutical mixture effects on microbial communities.

However, two concerns were highlighted by the predictive studies:
First of all, there was a strong hormesis effect caused by the PPCP mixture. When seen for the individual substances comprising the mixture, this violates the basic assumptions of the predictive concepts and no current strategy has been shown to amend this limitation. However, this was not seen in the concentration response curves of the mixture substances, and such unexpected mixture effects can by definition never be predicted. In this study, effect data was lacking for two of the comprising substances in the hormesis range of the mixture which might have indicated the observed hormesis of the mixture.

The second problem concerns the notion of using CA as the default concept for mixture toxicity predictions. The flat concentration response curves caused by the protein synthesis inhibiting antibiotics caused to IA predict a higher toxicity than CA, which is the reversed order compared to most other studies. Compared to previously published studies, there are indications that this is a common property of antibiotic effects on bacterial communities. Hence, this might cause problems if there are cases where CA does not provide a reasonable good estimate of mixture toxicity as it does in paper I.

8.5 Are interactions to be expected in pharmaceutical combinations?

There were no explicit reasons for expecting interactions in the pharmaceutical mixture in paper II, and none were seen. Antibiotic combinations have specifically been pointed out as prone to interact (Yeh et al 2006, Yeh et al 2009). However, no strong indications on interactions were seen in the predictive study in paper I. Even when trying to provoke synergistic effects by combining ciprofloxacin with chemosensitizers (paper IV), no increased toxicity beyond the predictable was seen. Even though much more information on this topic is needed before drawing any general conclusions, the results from this thesis provide no cause for concern.

8.6 Necessary improvements for increasing the ecological realism in environmental risk assessment of pharmaceuticals

To account for potential effects of pharmaceuticals in the aquatic environment on natural microbial communities, an addition to current environmental risk assessment strategies is needed. The assessment needs to be convenient, have high throughput and show high inter-lab reproducibility. There is no such thing as a standardized natural microbial community, and it is clear from these studies that

quite severe shifts in sensitivity can be seen between tests and seasons. Still, the same limitations have been shown for standardized test using sludge communities since they are commonly sampled from the STP most convenient to the investigator. Hence, using natural microbial communities for environmental risk assessment might entail neither more work nor higher uncertainties.

The endpoints assessed today is in most cases overarching parameters as growth or reproduction, which is of course a necessary parameter but might need to be amended also with more specific endpoints when it comes to risk assessment of pharmaceuticals. However, the ecological relevance of e.g. inhibition of protein synthesis in one or a few bacterial species must first be more thoroughly evaluated. In addition, if exposure in general to sewage treatment plant effluents may induce tolerance/resistance in the microbial communities, also such endpoints need to be considered.

One of the most important improvements needed in environmental risk assessment is the inclusion of mixture studies. This need has repeatedly been pointed out, and more and more evidence has been gathered to support that statement. The use of the predictive concepts CA and IA also for effect estimations of pharmaceutical mixture effects on natural microbial is supported by the findings in this thesis. The probability of interactions in mixtures have sometimes been advocated as reasons not to use predictive approaches, however this thesis have provided no support for that notion, not even when intentionally trying to provoke synergistic effects.

9 | Outlook and suggestion on further studies

Because of the increased access to advanced molecular tools, the immense diversity of the microbial communities in the aquatic environment is rapidly being unravelled, e.g. in the immense task of sampling in the Venter global ocean survey (see e.g. Shaw et al, 2008). In order to also protect this diversity and function in the same way as for organisms higher in the food web, their sensitivities and responses to contaminants such as e.g. pharmaceuticals needs to be much further investigated. Based on the results in this thesis, there are many issues that need to be investigated further. I have in the follow text selected a few, mainly based on personal interest.

In all studies in this thesis when exposing bacterial communities to antibiotics, the resulting concentration response curves have been extraordinary flat. This has both implications for risk assessment and mixture toxicity predictions, as previously discussed. However, if this is a general response of bacterial communities, for antibiotics and for all endpoints cannot be decided from the few studies in this thesis, but needs to be further investigated.

The effects of the pharmaceuticals tested in this thesis were mainly assessed on functional or semi-functional endpoints for bacteria and on pigment profiles for the algae. However, also molecular studies (e.g. genomics, proteomics and metabolomics) are needed to obtain a more encompassing estimation of the effects provoked. Hence, the combination of functional and molecular studies would be optimal, since they provide different kinds of information. This would be especially valuable when it comes to further investigations on resistance development by bacterial communities exposed to sewage treatment plant effluents. In this thesis a large increase in tolerance of the communities exposed to high concentrations of antibiotics, but the underlying mechanisms can mainly be speculated on without also studying the genetic information in the community.

Finally, there is a growing body of research on the ecological role of antibiotics that focus on the suggestion of antibiotics as signalling molecules instead of weapons. If this is truly the case, then low concentrations of antibiotics in the environment may cause effects on species interactions, with unknown consequences. Therefore, this would be an important issue also for microbial ecotoxicologists to adress.

Svensk populärvetenskaplig sammanfattning

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Under de senaste 100 åren har nya läkemedel revolutionerat vår möjlighet att behandla tidigare dödliga sjukdomar, vilket till viss del ligger till grunden för dagens västerländska samhälles förväntan på både livslängd samt fysisk och psykisk hälsa. När antibiotikan började användas i större skala i mitten 1900-talet, ansågs den vara en mirakelmedicin med förmågan att bota alla infektionssjukdomar. Det tog dock inte lång tid innan myntets baksida visade sig. Efter endast ett års klinisk användning av penicillin visade sig i stort sett alla bakterier av arten *Staphylococcus aureus* (en vanlig orsak till t.ex. matförgiftning, sårinfektioner, skelettinfektioner och infektion i hjärtklaffarna) återfunna på sjukhus i USA vara resistenta. De senaste årens utveckling av sk. superbakterier, dvs bakterier resistenta mot mer än en typ av antibiotika visar tydligt på en typ av negativa konsekvenser av vår höga läkemedelskonsumption.

En annan konsekvens är spridningen av aktiva substanser i miljön, vars effekter är fokus för denna avhandling. De läkemedel vi äter försvinner inte i kroppen utan kommer så småningom att utsöndras via urin och avföring. När läkemedel används inom djurhållning kommer utsöndringen att ske direkt i naturen, medan humanläkemedel utsöndras till avloppssystemet. Därför innehåller det avloppsvatten som når reningsverken vanligtvis en mängd olika läkemedel. Avloppsreningsverk är normalt sett inte designade för att rena bort syntetiska föroreningar utan för att minska utsläppen av näringämnen. Trots det tas en del av läkemedlen bort under reningsprocessen genom att fästa till partiklar i vattnet eller brytas ner genom kemiska eller biologiska processer. Till slut kommer dock ändå en viss mängd av läkemedlen att följa med genom hela reningsprocessen för att till slut följa med avloppsvattnet ut i naturen, där de sedan sprids vidare. Därför har läkemedel visat sig kontaminera de flesta vattenmiljöer från grundvatten till havsvatten, med högst koncentrationer återfunna i utlopp från avloppsreningsverk.

Läkemedel är substanser som aktivt påverkar levande varelser. Därför är det sannolikt att de också har förmågan att påverka de varelser som blir utsatta för dem i naturen. Detta gäller särskilt antibiotika som är avsedda att döda bakterier, oavsett om det är ”onda” bakterier som orsakar infektioner eller ”goda” bakerier som utför viktiga funktioner i naturen. De procedurer som används idag för att bedöma miljörisker av läkemedel är inte nödvändigtvis tillräckliga för att skydda även de mikroorganismer i naturen som utgör basen i näringsväven. I de fyra vetenskapliga artiklar som min avhandling bygger på har jag därför undersökt de eventuella riskerna som läkemedel i miljön utgör mot mikrobiella samhällen i både marina- och sötvattensmiljöer. Fokus har varit dels på studier av enskilda substanser, men framförallt på kombinationseffekter av läkemedel eftersom en blandning av giftiga ämnen generellt sett brukar orsaka en högre effekt än de ingående ämnena var för sig.

Det vore omöjligt att experimentellt testa effekter av alla möjliga blandningar i naturen. Alltså måste alternativ användas om detta ska fungera som ett sätt att utvärdera miljörisker. Ett sådant är att matematiskt förutsäga effekten av en blandning med hjälp av kända effekter av de ämnen som ingår i den. Två sådana matematiska koncept har föreslagits, Concentration Addition (CA) för blandningar av ämnen med lika verkningsmekanism och Independent Action (IA) för blandingar av ämnen som verkar på olika sätt. Dessa har visat sig fungera väl för att förutsäga effekter av blandningar på enskilda arter, men huruvida de också är användbara för effekter av läkemedelsblandningar på mikrobiella samhällen är okänt.

Dessa beräkningsmodeller förutsätter att de ingående substanserna i en blandning beter sig likadant oavsett om de är själva eller blandade, d.v.s. inga interaktioner får ske mellan blandningssubstanserna för att CA och IA ska gå att använda. Det har visats att det finns substanser i miljön som inte är giftiga i sig själva men som kan öka andra ämnens giftighet genom förhindra cellerna att göra sig av med dom, s.k. kemosensitiserande ämnen. Dessa skulle inte bara öka risken från läkemedel i miljön men också göra att beräkningsmodellerna blev verkningslösa.

De koncentrationer som normalt ses i naturen är låga, oftast några få miljondelar av en liter vatten. Däremot har dåligt kontrollerad produktion av generiska läkemedel i Indien visat sig släppa ut antibiotika i de omgivande vattnen, t.ex. av ciprofloxacin i samma höga koncentrationer som används för att bota bakterieinfektioner. Att dessa koncentrationer är extremt giftiga för de mikrober som lever i vattnen där utsläppen sker är inte svårt att föreställa sig, men det finns också en hög risk för utveckling av bakterieresistens i floden som tar emot avloppsvattnet.

Mina resultat visar att trots att de låga nivåer vi ser i miljön av läkemedel, så är de inte utan risk. Effekter av antibiotikan klortetracyklin (artikel I) kan ses redan vid koncentrationer som återfinns i avloppsvatten från reningsverk idag, och när 5 fem läkemedels-, hygien- och hushållsprodukter blandades i koncentrationer som inte individuellt orsakade någon signifikant effekt, orsakade blandningen nästan 30% effekt. De blandningseffekter som undersöktes (artikel I och II) var möjliga att beräkna med antingen CA eller IA, och även när kemosensitiserande ämnen blandades med antibiotikan ciprofloxacin blev inte effekten högre än beräknat. När avloppsvatten från fabrikerna i Indien testades visade det inte bara vara extremt giftigt som väntat, men också orsaka en ökad tolerans mot ciprofloxacin hos de utsatta mikrobiella samhällena. Om detta berodde på resistensutveckling eller inte kan dock inte avgöras utan vidare studier.

Sammanfattnigsvis så visar studierna i min avhandling att de strategier som används idag för att bedöma risker av läkemedel i miljön inte är tillräckliga för att skydda mikrobiella samhällen i naturen utan behöver förstärkas. Framförallt måste effekter av blandingar av läkemedel inkluderas för att spegla den reella exponeringssituationen.

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”It was the best of times, it was the worst of times...”
Nu skrev förvisso Charles Dickens inte om att vara forskarstuderande, men för mig beskriver citatet väldigt väl hur jag har upplevt tillvaron de senaste fem åren. Mest har det varit ”best of times”, även om den senaste månadens slit med att få klart avhandlingen definitivt bitvis faller under den andra beskrivningen. Att jag har trivts så bra med att doktorera är det många som förtjänar ett tack för att ha bidragit till.

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Åsa: du tog hand om en förskrämd nybliven doktorand för fem år sedan och har varit en klippa (oavsett vad det gällt) sedan dess. Har saknat dig på IVM det senaste! Vi fortsätter väl ringas vid varje vecka för att utbyta det senaste...? **Tobias:** probably the best fältarbeteskamrat in the world. Du förstår vikten av indisk mat, har lärt mig uppskatta ale och helt enkelt varit ett väldans trevligt sällskap genom åren oavsett om det gällt bad i Mölndalsån, lunch eller after work. **Martin:** min molekylära vän i det ekologiska träsket, tillika min personliga hejarklack det senaste året. Hoppas du vet hur mycket jag har uppskattat det! Du har varit gått sällskap på alla möjliga mindre och större äventyr, som t.ex. den beryktade sälutflykten, för att inte tala om ett givet lunchsällskap. **Cissi:** trots en gedigen insats på osmoslabben kan väl ingen kalla oss arbetskamrater. Våra kaffepromenader räddade mitt huvud från att explodera, och som en given partner in slarv får jag tacka för grymt mycket kul genom åren (nog så viktigt för att överleva doktorand-tiden!). **Ida** och **Annelie:** tänk så mycket bättre det hade varit (åtminstone för mig) om ni inte envisas med att vara mest på Kristineberg (F´låt, Sven Lovén Center.

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Sannolikt va, det betyder väl nåt som är likt sanning.
Men riktigt lika sant som sanning är det inte om det är sannolikt.
Nu har vi tydligen inte råd med äkta sanningar längre,
utan vi får nöja oss med sannolikhetskalkyler.
Det är synd det, för dom håller lägre kvalitet än sanningar.

Tage Danielsson,
ur ”Under Dubbelgöken” (1979)